Award Number: W81XWH-13-1-0106

TITLE: Functional Characterization of CENP-A Post-Translational Modifications in Chromosome Segregation

PRINCIPAL INVESTIGATOR: Sathyan Kizhakke Mattada

CONTRACTING ORGANIZATION: University of Virginia
Charlottesville, VA 22904-4195

REPORT DATE: September 2016

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
              Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
September 2016	Final	15Jun2013 - 14Jun2016

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Functional Characterization of CENP-A Post-Translational Modifications in Chromosome Segregation	W81XWH-13-1-0106

5b. GRANT NUMBER
W81XWH-13-1-0106

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)	5d. PROJECT NUMBER
Sathyan Kizhakke Mattada	

5e. TASK NUMBER

5f. WORK UNIT NUMBER

E-Mail: sk8fz@virginia.edu

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
University of Virginia 1001 N Emmet ST. Charlottesville VA 22903-4833	

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012	

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Colorectal cancer is the second leading cause of cancer death in the United States. Approximately 85% of colorectal cancers are CIN+ (Chromosomal instability) and are associated with poor survival. The molecular mechanisms responsible for the CIN phenotype and hence means to target this pattern of genome instability remains poorly defined. I hypothesized that, post-translational modifications (PTM) of the centromeric nucleosome, specifically on the histone variant, CENP-A, will direct centromere activity, and that perturbations of such could lead to aneuploidy and cancer. We proposed to decipher the pathway that leads to CENP-A α-amino methylation and to determine the function it plays in ensuring the fidelity of chromosome segregation. We have shown here that CENP-A is methylated by NRMT1 both *in vitro* and *in vivo*. CENP-A is methylated before it is deposited into the centromere and that methylation persists throughout the cell cycle. We also found that CENP-A methylation required for cell survival. We established that CENP-A α-amino tri-methylation required for ensuring high fidelity of chromosome segregation by recruiting CENP-T and CENP-I into centromere. In the absence of methylation, the centromere localization of these proteins decreases which reduce the localization of the NDC80. NDC80 connects the kinetochore to the spindle pole. We found that tumor suppressor p53 plays a role in monitoring the accuracy of the chromosome segregation and formation of bipolar spindle. In the absence of p53, loss of CENP-A methylation leads to multipolar spindle formation. This triggers chromosomal instability and evolution of aggressive tumors. Importantly, we found that loss of CENP-A α-amino tri-methylation trigger a proliferation advantage and cells form bigger colonies in colony formation assay. Suggesting the evolution of aggressive clones. In summary, we have identified a novel pathway where centromere/kinetochore components act together with p53 to prevent the evolution of the CIN tumors. Suggesting α-amino tri-methylation of CENP-A is an important post-translational modification necessary for maintaining accuracy of chromosome segregation.

15. SUBJECT TERMS
Colorectal cancer, post-translational modifications, CENP-A, NRMT1, centromere, CENP-A α-N-me3

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U	UU	57	19b. TELEPHONE NUMBER *(include area code)*

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Introduction:

Colorectal cancer is the second leading cause of cancer death in the United States[1]. Chromosomal instability (CIN) and microsatellite instability (MIN) are two major molecular hallmarks of colorectal cancer[2,3]. 85% of colorectal cancers are CIN+ and are associated poor survival[3]. The molecular mechanisms responsible for the CIN phenotype and hence means to target this pattern of genome instability in colorectal tumors remains poorly defined. I hypothesized that, similar to the nucleosomes of general chromatin, post-translational (PTM) modifications of the centromeric nucleosome, specifically on the histone variant, CENP-A, will direct centromere activity, and that perturbations of such could lead to aneuploidy and cancer. However, the identification of CENP-A PTMs has lagged behind PTMs of other histones because of the lack of a good purification strategy. Using a novel purification strategy we have identified three PTMs on the CENP-A tail, α-amino tri-methylation of initial Glycine, and phosphorylations at S16 and S18[4]. Previously, another phosphorylation was reported at S7[5]. We proposed to decipher the pathway that leads to CENP-A α-amino methylation (CENP-A α-N-me3) and to determine the function it plays in ensuring the fidelity of chromosome segregation. We also proposed to determine how its abrogation may cause carcinogenesis and ask whether targeting this PTM is a viable strategy to target colorectal cancer cells. Overexpression of CENP-A in colorectal cancer leads to its mislocalization to chromosome arms resulting in aneuploidy. Until now the only way to inhibit CENP-A function was through shRNA knockdown. However, understanding CENP-A amino terminal tail modification will provide enzymatic and therefore potentially druggable targets to inhibit this pathway.

In the first year report (report attached) we have demonstrated that CENP-A is methylated by NRMT1 both *in vitro* and *in vivo*. CENP-A is methylated before it is deposited into the centromere and that the methylation persists throughout the cell cycle. However, we found an increase in CENP-A methylation during prophase of the cell cycle. We established that CENP-A α-amino tri-methylation required for ensuring high fidelity of chromosome segregation, and hence preventing aneuploidy and cancer. Importantly, we found that loss of CENP-A α-amino tri-methylation in colorectal cancer cells trigger a proliferation advantage and they form bigger colonies in colony formation assay. Suggesting α-amino tri-methylation of CENP-A is an important post-translational modification necessary for maintaining accuracy of chromosome segregation.

In the second year (report attached) we have made progress in further elucidating the role of CENP-A α-amino tri-methylation. Using a CENP-A knockout cell line we have found the CENP-A α-amino tri-methylation contribute to cell survival. In the absence of α-amino tri-methylation, the cells undergo senescence. CENP-A methylation required for the formation of Constitutive Centromere Associated Network (CCAN) complex. We have found in the absence of CENP-A methylation the localization of CENP-T is reduced. This alteration might be the reason for the chromosome segregation defect we have seen in the methylation mutant cancer cells. We inferred that the impaired formation of kinetochore might leads to an imbalance in the force generated by the motor protein on the chromosomes that leads to multipolar spindle in CENP-A methylation mutant cancer cells. Hence, we partially inhibited one of the motor proteins

Eg5 using low concentration of monastrol and we were able to rescue the multipolar spindle formation. We conclude that CENP-A α-amino tri-methylation required for kinetochore formation and maintenance of bipolar spindle.

This year we have demonstrated that by completely replacing wild type CENP-A with methylation resistant mutant CENP-A in normal immortalized RPE CENP-A knockout cells, the loss of methylation required for normal chromosome segregation. The loss of methylation leads to reduced localization of CENP-T at the centromere and causes lagging chromosomes. We also established the role of p53 in forming multipolar spindle in the absence of CENP-A methylation. In the presence of p53 in RPE cells where endogenous CENP-A is replaced by methylation mutant CENP-A, we did not see significant increase in multipolar spindle formation. However, when we knockdown p53 in these cells we found that percentage of cells with multipolar spindle significantly increased similar to cells without active p53. Suggesting that in the absence of p53, the loss of CENP-A methylation is a contributing factor for carcinogenesis, which we have observed in our colony formation assay as well. We have submitted our finding to the journal 'Nature Communications'.

Keywords: Centromere, CENP-A, CENP-A α-amino tri-methylation, NRMT, CENP-T, CENP-C, Colorectal cancer, p53, Multipolar spindle, tumor

Overall project summary:
In this section we discuss our results that we accomplished during last year. Then we will discuss the importance of our overall findings in discussion part, and finally we will explain major materials and methods we used.

Results

CENP-A α-amino methylation facilitates the recruitment of CENP-T and CENP-I. To determine whether CENP-A methylation contributes to CCAN formation we examined the recruitment of CCAN proteins to the centromere in the RPE1 CENP-A$^{-/F}$ cells that expressing eGFP tagged CENP-A wild type or methylation mutants containing the CENP-A or H3 carboxyl termini. There was no difference in the recruitment of the CCAN proteins CENP-B, CENP-C, CENP- I, and CENP-T to centromeres between cells expressing wild-type CENP-A and CENP-A methylation mutants when endogenous CENP-A was present (Fig.1A- F).

However, when the endogenous CENP-A was removed by Cre-recombinase, we observed significant differences in CCAN recruitment after 5 days depending on the C-terminal sequence and alpha-amino terminal methylation status of CENP-A (Fig. 2A). CENP-C levels were significantly reduced in cells rescued with CENP-A containing the H3 C-terminus (^{WT}CENP-A^{CH3}) (Fig. 2B,C) consistent with a direct role for the C-terminus in recruiting CENP-C as shown previously[6,7]. The amino terminus of CENP-A also plays a role in CENP-C recruitment via its binding with CENP-B[8]. However, the methylation of CENP-A is not involved in this process, since we observed no difference in CENP-C recruitment between wild-type (^{WT}CENP-A) and CENP-A methylation mutants (^{MT1}CENP-A), irrespective of the C-terminal tail.
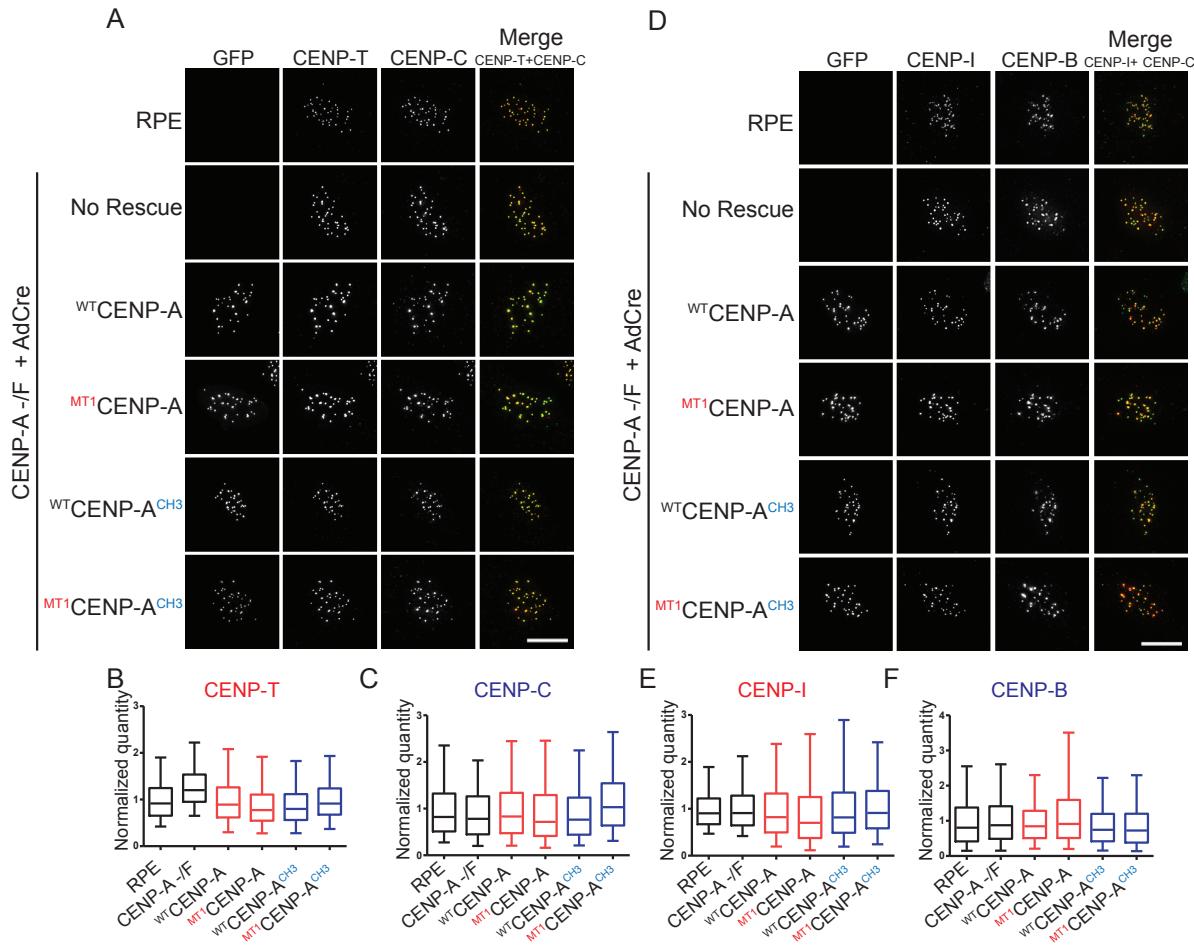
Figure 1. CCAN components did not change drastically in cells expressing wild type or mutant exogenous CENP-A in the presence of endogenous CENP-A (A). Representative images of the cells stained for CENP- T and CENP-C, (B&C). Quantitation of CENP-T and CENP-C respectively at the centromere in cells expressing endogenous CENP-A, (D). Representative images of the cells stained for CENP-I and CENP-B, (E&F). Quantitation of CENP-I and CENP-B respectively at the centromere. Box-and-whisker plots. Central lines, medians; whiskers, range 5-95 percentile; outliers not shown, Scale bar, 10 µm, (G). Localization of CENP-T, CENP-I, CENP-C and CENP-B at the centromere after replaced with CENP-A N-terminal tail deletion mutant.

In contrast, CENP-T and CENP-I localization at the centromere was significantly reduced in CENP-A methylation mutants (^{MT1}CENP-A) relative to wild-type expressing cells (^{WT}CENP-A) (Fig. 2A-G). The reduction of CENP-T and CENP-I was observed in methylation mutants compared to the wild-type N-terminus when the endogenous C-terminus was present (^{WT}CENP-A vs. ^{MT1}CENP-A), and when the H3 C-terminal chimeras where expressed (^{WT}CENP-A^{CH3} vs. ^{MT1}CENP- A^{CH3}). A reduction of CENP-I and CENP-T centromeric levels was also observed when comparing ^{WT}CENP-A expressing cells with ^{WT}CENP-A^{CH3}. Therefore CENP-T and CENP-I are recruited via two independent pathways, through the C-terminal tail—possibly through CENP-C—and by an N-terminus dependent pathway that requires alpha-amino terminal methylation.
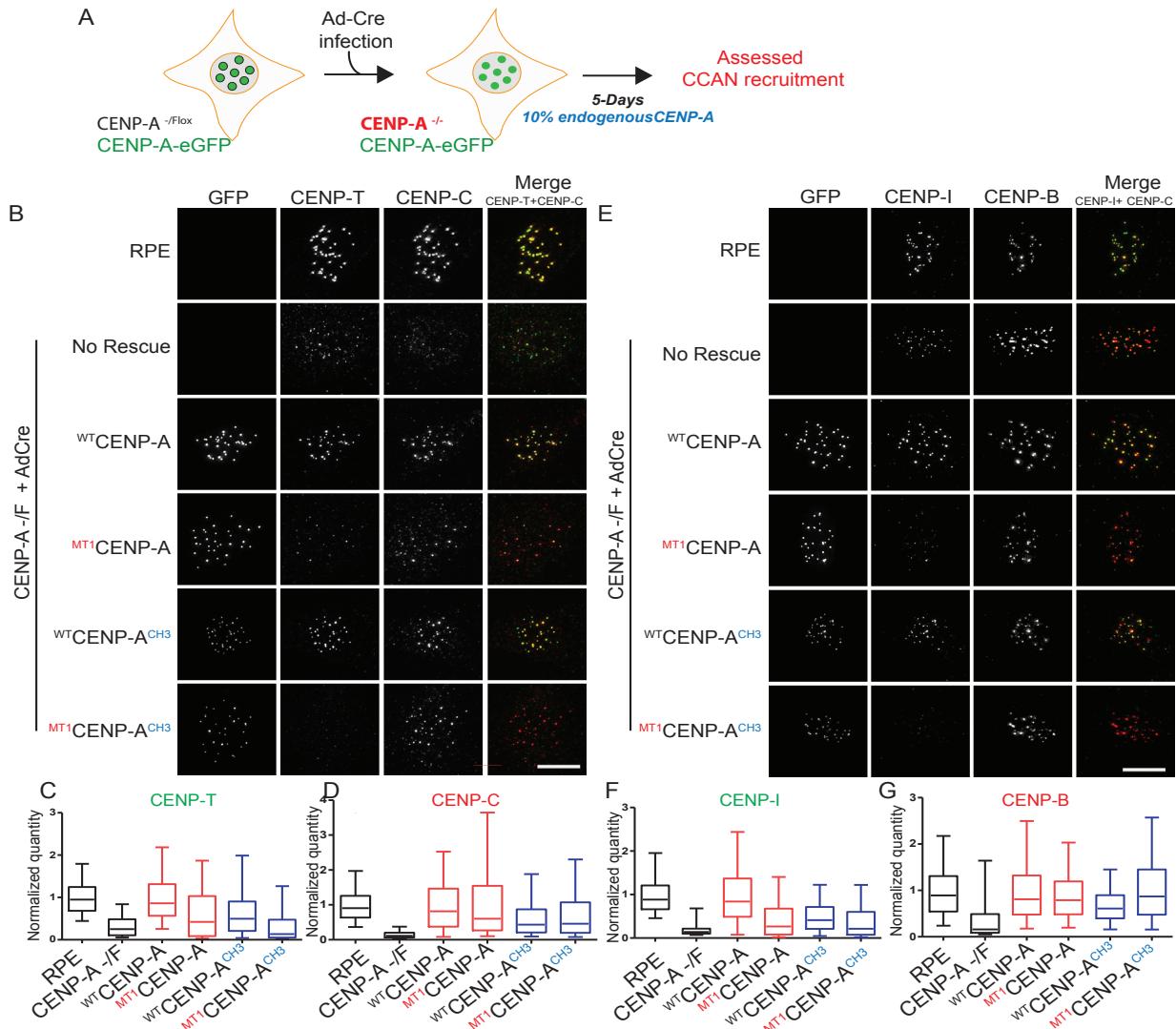
Figure 2. CENP-A α-amino methylation is required for CCAN formation. (A).Schematic of the experiment, (B). Representative images of the cells immune-stained for CENP-T and CENP-C. Centromeric CENP-T was decreased upon CENP-A knockout and is rescued by replacement of the wild type CENP-A but not with methylation mutant, (C&D). Quantitation of CENP-T and CENP-C at the centromere after removal of endogenous CENP-A by cre-recombinase, (E). Representative images of cells immune-stained for CENP-I and CENP-B. CENP- I level decreased when endogenous CENP-A was removed by Cre expression and is rescued by replacement with the wild type CENP-A but not with methylation mutant, (F&G). Quantitation of CENP-I and CENP-B at the centromere after removing endogenous CENP-A by cre-recombinase. Box-and- whisker plots. Central lines, medians; whiskers, range 5-95 percentile; outliers not shown, Scale bar, 10 µm.
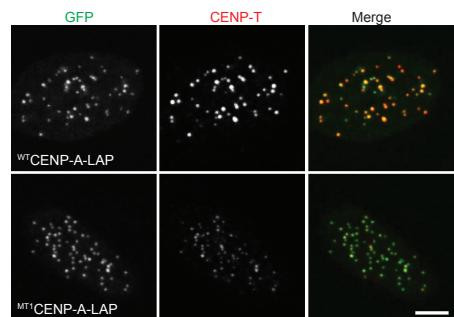
**Figure 3. CENP-A α-amino methylation is required for CCAN formation.** Representative images of the cells immune-stained for CENP-T. Centromeric CENP-T was decreased in CENP-A methylation mutantreplaced cells aster endogenous CENP-A is suppressed by shRNA.

CENP-A α-amino tri-methylation required for accurate chromosome segregation in normal immortalized RPE cells

In order to determine if the impairment of the CCAN

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caused by lack of CENP-A methylation reduces the kinetochore function in normal cells, the cells were treated with 0.1µm nocodazole for six hours and then released for one hour to progress into anaphase and examined for lagging chromosomes (Fig. 4A). Cells expressing the CENP-A methylation mutant were significantly more likely to contain lagging chromosomes compared to wild type CENP-A replaced cells and parental RPE-1 cells (Fig. 4B,C). Furthermore we found that loss of CENP-A methylation resulted in a significantly higher number of micronuclei even without nocodozole treatment (Fig. 4D). In summary, CENP-A methylation is required for CCAN formation and faithful chromosome segregation. To see how loss of CENP-T in CENP-A methylation mutant cause chromosome segregation defect, we stained the cells after replacement of the mutant or wild-type CENP-A with NDC80 (Fig. 4E). We found significant reduction in NDC80 at the kinetochore during mitosis (Fig. 4F). NDC80 connects centromere/kinetochore to the spindle and helps the cells to segregate chromosomes. The study, thus, established that CENP-A methylation required for the accurate formation of the centromere and kinetochore and maintaining the high fidelity of chromosome segregation.
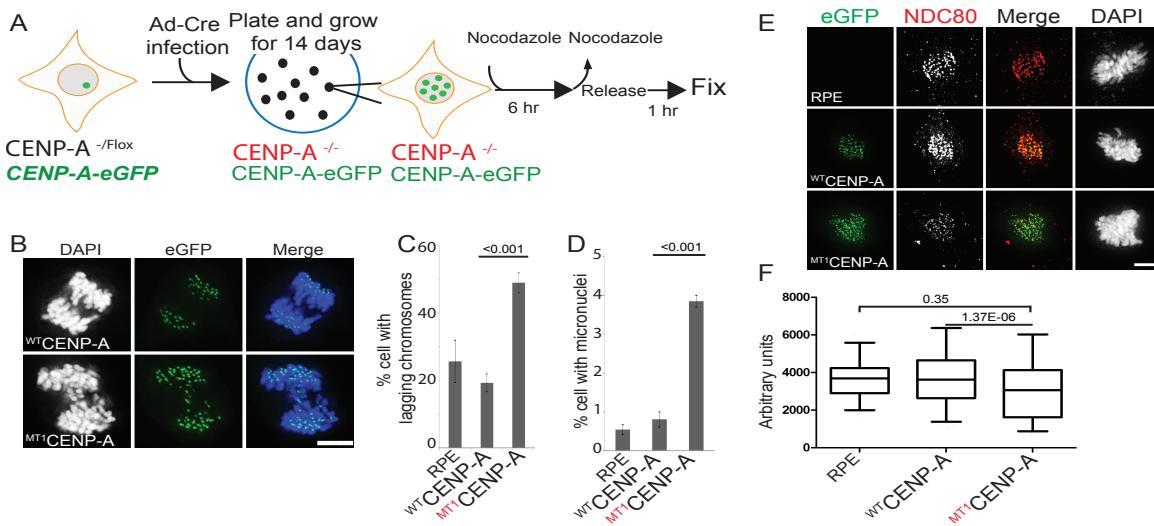

Figure-4. CENP-A α-amino tri-methylation required for accurate chromosome segregation in normal immortalized RPE cells (A). Schematic of the experiment in B-C. eGFP tagged CENP-A wild type and mutant integrated RPE-CENP-A -/F cells were infected with cre- recombinase virus to remove endogenous CENP-A. Individual colonies were picked after 14 days and expanded. The cells were treated with 0.1mg/ml Nocodazole for 6 hours and then released for one hour, fixed and DAPI stained (B). Cells expressing the methylation mutant showed significantly high percentage of (C) lagging chromosomes and (D) micronuclei formation, (E). CENP-A wild-type or mutant replaced RPE cells stained for NDC80, (F). The Box and Whisker plot showing the quantitation of NDC80 localization to the kinetochore. The NDC80 level significantly reduced in the mutant.

Loss of p53 along with loss CENP-A α-amino tri-methylation causes multipolar spindle formation

To see the role of CENP-A methylation in maintaining bipolar spindle and how p53 plays a role in this process, we made complete replacement of the endogenous CENP-A either with ^{WT}CENP-A or ^{MT1}CENP-A in RPE-CENP-A$^{-/F}$ cells (Fig. 5A). We knocked down p53 in these cells using shRNA. We found that in the presence of p53, there is no significant difference in multipolar cells in wild type and mutant CENP-A cells. But when we knockdown p53, the multipolarity increased in methylation mutant but not in CENP-A

wild-type replaced cells (Fig5 B-D). This directly shows loss of CENP-A methylation leads to multipolar spindle formation in the absence of p53. Suggesting a synergistic effect of both these events in tumorigenesis.

CENP-A α-amino tri-methylation prevents multipolar spindle formation by recruiting CENP-T

To see whether the multipolarity caused by the CENP-A methylation mutant is due to CENP-T or CENP-I loss, we knocked down CENP-T or CENP-I in HeLa TRex cells (Fig.6 A). Surprisingly, we found that only the CENP-T knockdown cause multipolarity but not CENP-I, suggesting α-amino tri-methylation of CENP-A prevents multipolarity by recruiting CENP-T. Since the methylation mutant cause multipolarity only in p53 null cells, we checked the same true for CENP-T knockdown. We found that only in p53 null HCT116 cells, the knockdown of CENP-T cause significant increase in multipolar spindle formation (Fig.6 B). These results again confirm the role of p53 in preventing chromosome instability in centromere/kinetochore defective cells.

Discussion:

The post-translational methylation of alpha-amino groups was first discovered over 30 years ago[9,10]. However, its biological function remains obscure except in the case RCC1 (Ran guanine nucleotide-exchange factor) where it is required for its



Figure-5. Loss of p53 along with loss CENP-A α-amino tri-methylation causes multipolar spindle formation
(A). Schematic of the experiment. eGFP tagged CENP-A wild type and mutant integrated RPE-CENP-A -/F cells were

localization into chromosomes[10]. We identified a novel function of alpha-N-methylation. We found that alpha-N-methylation of CENP-A is required for maintaining genomic stability and its loss leads to aneuploidy. We demonstrated that CENP-A is methylated throughout the cell cycle and methylated CENP-A increases during prophase. Reduction in CENP-A level increases the propensity of multipolar spindle pole formation and lagging chromosome in p53 null colorectal cancer cell HCT116. Where as in isogenic p53+/+ cells there is no significant increase in multipolar spindle formation after CENP-A knockdown. In our knockdown replacement experiment, wild-type CENP-A stably integrated cells had similar percentage of multipolar cells as that of parental cell lines both in colorectal cancer cell HCT116 p53-/- and HeLa. But the methylation mutant CENP-A stably integrated cells showed significantly increased multipolar spindle in p53-/- HCT116 and p53 inactive HeLa cells suggesting CENP-A methylation mutants are hypomorphic. We experimentally proved the role p53 in the formation of multipolar spindle in the absence of CENP-A methylation. When we knockdown p53 in the RPE cells with wild-type CENP-A, there is no significant increase in multipolar spindle formation.
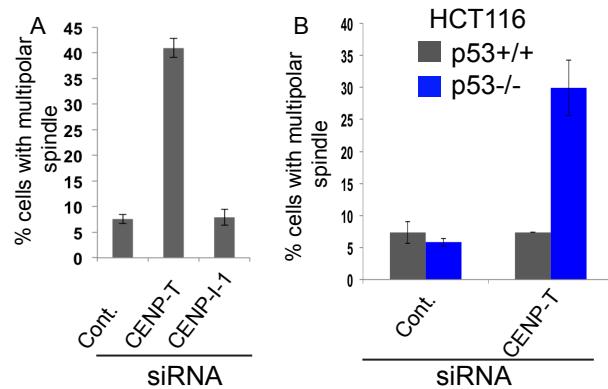
Figure-6. Loss of CENP-T causes multipolar spindle formation in a p53 dependent manner similar to loss CENP-A α-amino tri-methylation. (A). Knockdown of CENP-T in HeLa TRex cells showing increased multipolar spindle formation but not in CENP-I knockdown cells, (B). HCT 116 cells without p53 showing multipolar spindle formation upon CENP-T knockdown but not in p53+/+ cells. Suggesting in the absence of p53, CENP-T knockdown cause multipolar spindle formation and chromosome instability.

However, when we knocked down p53 in CENP-A methylation mutant cells, there was a significant increase in multipolar spindle formation in the cells. Suggesting that loss of p53 and CENP-A methylation has synergistic effects in carcinogenesis. Similar to this finding we found that cells forms larger colonies in cells with reduced CENP-A methylation in the absence of p53. We also analyzed chromosome missegregation defects in these cells and found that there is an increase in lagging chromosomes compared to wild-type irrespective of p53 status. We have established that loss CENP-A methylation in cells with p53 induces chromosome segregation defects and such cells may be eliminated by inducing senescence. However, when p53 is also lost in the absence of CENP-A methylation, the cells undergo further transformation. Suggesting that CENP-A methylation has a protective effect in tumorigenesis.

We have identified that loss of CENP-A methylation leads to defects in the formation of Constitutive Centromere Associated Network (CCAN) complex at the centromere. The centromere localization of CENP-I and CENP-T is significantly reduced in methylation mutant cells. This suggests that CENP-A methylation is partially involved

in the localization of these proteins to the centromere. Such changes in CCAN might be the reason for the chromosome segregation defects and multipolar spindle in the CENP-A methylation mutant cancer cells due to defective kinetochore formation.

Overall we have discovered that CENP-A methylation contributes to cell survival and in the absence of methylation cells undergo senescence. However, in the absence of p53 loss of CENP-A methylation causes multipolar spindle formation and confer more tumorigenic potential to the cancer cells. We have found that CENP-A methylation required for the formation of CCAN complex at the centromere and in the absence of that cells undergo missegregation of their chromosomes, which is the basis of chromosome instability. We established a novel function of the α-amino tri-methylation of CENP-A in the formation of centromere and kinetochore and accurate segregation of the genetic materials. Moreover, we established that centromere/kinetochore defects in the absence of p53 induce chromosome instability (CIN), which is present in 80% of colorectal cancer. Thus the present study identified how CIN tumors are developed.

Materials and methods:
Creation of CENP-A complete replacement RPE cells: RPE CENP-A knockout cell line generated by Don Cleaveland Lab[7] used for this study. In this cell lines one allele of CENP-A is knocked out and the other allele is a Flox allele, which upon Ad-cre recombinase virus infection will be removed. We made CENP-A wild type and mutant stable lines in these cells. The endogenous CENP-A was then removed by infecting the cells with Ad-cre for four hours and then the virus was washed out. 48 hours after infection, 500 cells were split into 10cm plates. The colonies were selected after 14 days and screened for complete replacement of endogenous CENP-A by western blotting.

Nocodazole treatment: The cells were treated with 0.1mg/ml Nocodazole for 6 hours and then released for one hour. Cells were then fixed and stained for DAPI. Chromosome segregation defects were counted in mitotic cells.

Immunofluorescence: Cells were either pre-extracted with 0.1% triton in 1X PBS for 3 minutes and then fixed in 4% formaldehyde for 10 minutes. The cells were then blocked in 2% BSA and 2% fetal calf serum with 0.1% triton in 1XPBS. It was incubated with primary antibodies one hour and then secondary antibody was added for one hour. All washes between each step were done in 1XPBS + 0.1% triton. DNA was stained with DAPI and were mounted in prolong. Cells were examined and images were acquired using a Delta Vision optical sectioning deconvolution instrument (Applied Precision) using a X100 oil-immersion Olympus objective lens connected with Photometrics CoolSNAP HQ camera and Softwrox acquisition software. Images were deconvolved and presented as maximum stacked images. The antibodies used for immunofluorescence were: mouse anti-CENP-A (1:1000), Rabbit anti-me3CENP-A (1:200), Rabbit anti-CENP-T (1:1000), Mouse anti-CENP-C (1:1000), Rabbit anti-CENP-I (1:1000, Mouse anti-CENP-B (1:250), Mouse anti-α-tubulin (1:1000).

Following antibodies were used for western blotting. Mouse anti-p53 (DO7, Santa Cruz) (1:1000), Mouse anti-tubulin (1:1000).

Key accomplishments:

Statement of Work (SOW) nutshell

We accomplished almost all the stated goals and we submitted a manuscript regardings these findings to 'Nature communications'.

Specific Aims

AIM1: Determine the cell cycle dynamics and pathway responsible for α-N-me3 of CENP-A

    1.1   Analyze cell cycle dependent regulation of CENP-A α-N-me3

        1) Cell cycle distribution of CENP-A methylation in HeLa, U2OS, HCT116 and RPE1 cells will be studied by immunofluorescence. **Accomplished First year**

        2) CENP-A stability and methylation in SNAP tagged CENP-A HeLa stable cell line by TMR staining living cells and follow the stability of TMR stained CENP-A with and without NRMT knock down over cell divisions. **Accomplished First year**

        3) The pre-nucleosomal and nucleosomal CENP-A will be analyzed in HeLa cells by fractionating cells and western blotting for CENP-A and methylated CENP-A. **Accomplished First year**

    1.2   Identification of a pathway responsible for α-N-me3 of CENP-A

        1) NRMT methylate CENP-A *in-vitro*. Knock down NRMT by shRNA in HeLa cells and stain for methylated CENP-A both by immunofluorescence and western blotting. **Accomplished First year**

        2) Asses total CENP-A levels after NRMT knock down to know the stability of methylated CENP-A in HeLa cells. **Accomplished First year**

    1.3   Analyze the status of CENP-A methylation in cancer and its role in colorectal cancer progression

        1) We will use the CIN+ cell lines like HT-29 and MIN+ cell lines like HCT-116 to see any difference in overall methylation between MIN and CIN cell lines by immunofluorescence.

        2) To see the role of CENP-A methylation in colorectal cancer progression we will use NCI, cancer diagnosis program (CDP) colon tissue microarray. These TMAs are designed to evaluate the markers with tumor stage, clinical outcome and other clinic-pathologic variables.

        These TMA contains 367 invasive colon cancer, 34 adjacent normal tissue, 40 normal colon, 37 Adenomatous polyps, 32 non-colon normal tissues and 48 cell lines. The TMA was designed by NCI statisticians for high power for comparison of marker values. **Since the antibody generated against methylated CENP-A was found to recognize a major non-specific band in the western blots, we could not do tissue level staining.**

AIM 2: Identify the functional consequence of CENP-A α-N-me3

    2.1   Delineate the role of CENP-A α-N-me3 on cell viability and cell cycle progression

        1) HeLa and HCT-116 CENP-A non-methylatable stable lines will be used to understand role CENP-A   methylation in cell viability and cell cycle

progression. **Accomplished second year. Found that CENP-A methylation is required cell survival.**

2.3   Delineate the role of CENP-A α-N-me3 in centromere activity and or kinetochore formation
1) These studies will be done in HeLa cells. We do peptide pull down from HeLa extracts and confirmation in HeLa cells. **Accomplished second and third year. We found that CENP-A methylation is required CENP-T and CENP-I recruitment and NDC80 localization to the kinetochore.**

2.4   Evaluate how PTMs of CENP-A influence CIN and MIN in colorectal cancer cell survival
Accomplished. Found that loss of CENP-A methylation gives a proliferative advantage for the cells and the cancer cells grows faster.

Following are the key accomplishments of the proposed work

Year I (First year report attached)

1. Identified NRMT1 methylates CENP-A at its α-amino terminal.
2. CENP-A is methylated throughout the cell cycle.
3. Pre-nucleaosomal CENP-A is methylated.
4. We made several methylation resistant CENP-A mutants.
5. Loss of CENP-A cause aneuploidy by forming multipolar spindle and missegregation of chromosomes.
6. α-amino tri-methylation of CENP-A ensures high fidelity of chromosome segregation. Its loss cause multipolar spindle and lagging chromosomes in p53 null background and lagging chromosomes in the presence of p53. Both these alterations fundamentally cause aneuploidy, a form of genetic alterations prevalent (85%) in colorectal cancer.
7. We also found that methylation mutant CENP-A forms bigger colonies in colony formation assay suggesting role of methylation in controlling proliferation.

Year II (Second year report attached)
1. CENP-A α-amino tri-methylation required for the survival of the cells.
2. Loss of α-amino tri-methylation of CENP-A leads to senescence in normal cells.
3. Loss of CENP-A induces p53 and that leads to senescence. This explains the increased tumorigenic potential seen in CENP-A methylation mutant, which is less functional, in p53 null cells.
4. α-amino tri-methylation of CENP-A required for the localization of CCAN components to the centromere and centromere homeostasis.
5. Force imbalance generated by the improper kinetochore formation and resultant misaligned chromosomes may be the reason for the multipolar spindle formation in p53 null CENP-A mutant cell lines.

Year III

1. CENP-A methylation required for accurate segregation of chromosomes in normal cells.
2. The CENP-A methylation is required for the formation of constitutive centromere associated complex.
3. Discovered that CENP-A methylation functions in the recruitment of CENP-T and CENP-I proteins.
4. Discovered that loss of CENP-T leads to multipolar spindle similar to loss of CENP-A methylation, suggesting that CENP-A methylation preventing multipolar spindle formation through CENP-T.
5. Discovered that loss of CENP-A methylation reduce CENP-T at the centromere which in-turn reduce NDC80. The key molecule which link kinetochore to poles.
6. Established that in the absence of p53, loss of CENP-A methylation leads to multipolar spindle formation and defective chromosome segregation.
7. Discovered a novel mechanism how CENP-A methylation cooperates with p53 to prevent chromosome instability. In the absence of p53, a small change in centromere/kinetochore components causes aneuploidy and tumor evolution and aggressive tumor.

Conclusion:

We have made significant progress in elucidating the function of α-amino tri-methylation of CENP-A. We achieved most of the proposed aims. We found that CENP-A is methylated by NRMT1 and this modification persists throughout the cell cycle. We also found that α-amino tri-methylation of CENP-A is critical in orchestrating chromosome segregation and its abrogation may leads to aneuploidy and cancer. We also found that this modification required for the survival of the cells and in the absence of this modification, normal cells undergo senescence. We were able to show that α-amino tri-methylation of CENP-A is required for the efficient recruitment of CCAN proteins at the centromere. We discovered a novel mechanism how CENP-A methylation cooperates with p53 to prevent chromosome instability. In the absence of p53, centromere/kinetochore defects cause aneuploidy and tumor evolution and aggressive tumor. The accurate formation of CCAN and kinetochore is essential for high fidelity chromosome segregation. The manuscript regarding CENP-A methylation is udder revision in the journal 'Nature communications'.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

The presenting author underlined

FIRST YEAR;

Cancer Epigenetics, Keystone symposia, February 4-9, 2014.

N-terminal α-amino trimethylation of centromere histone CENP-A required for maintaining bipolar spindle and regulated cell proliferation. **<u>Kizhakke M. Sathyan</u>**, Aaron O. Bailey, Tanya Panchenko, Donald F. Hunt, Ben E Black, Daniel R. Foltz. Cancer Epigenetics, Keystone symposia, February 4-9, 2014.

Abstract of the poster:

The deposition of histone H3 variant CENP-A marks centromere in higher eukaryotes, chromosomal loci required for accurate segregation of chromosome. It has been shown a majority of cancers exhibits chromosomal instability, however, the mechanism is unclear. Overexpression of CENP-A and its chaperone HJURP occurs in many cancers and is sufficient to cause chromosome missegregation in cell culture. The posttranslational modification (PTM) of histones resulting in the recruitment of activator and repressive complexes to chromatin is a well established and broadly used method to regulate chromatin activity by the cell. We propose that the PTMs of CENP-A have a major role in preventing chromosomal instability. Despite the identification of nearly 14 PTMs on the histone H3 amino terminus, many of which have significant functional consequences, prior to our work only a single phosphorylation on serine 7 (pSer7) of CENP-A has been described in higher eukaryotes. Recently, we have reported three new PTMs on human CENP-A N-termini using high-resolution MS: trimethylation of Gly1 and phosphorylation of Ser16 and Ser18. The nascent N-terminal residue Gly1 becomes trimethylated on the α-amino group following initial methionine removal. Here we demonstrate that the N-terminal RCC1 methyltransferase (NRMT1) methylates CENP-A both *in vitro* and *in vivo*. Even though it was identified 30 years ago, the function of N-terminal α-amino group methylation is poorly understood. Methylation occurs in the prenucleosomal as well as nucleosomal forms of CENP-A, although nucleosomal CENP-A showed higher methylation. *In vivo* as well as *in vitro* data suggest that the amino terminal tail of CENP-A is sufficient for trimethylation. We also found significant differences in methylation of centromeric CENP-A during prophase of the cell cycle. The CENP-A methylation resistant mutants show multipolar spindles, multiple centrioles and multinucleated cells indicating a failure of cytokinesis in p53 inactivated HeLa cells. We also found similar results in p53-/- HCT116 cells. The methylation mutant cells formed larger and higher number of colonies indicating uncontrolled growth in p53-/- cells, where as flatter cells in p53+/+ cells may be due to induction of senescence. We are currently analyzing how the loss of CENP-A α-amino trimethylation leads to multipolar spindles and uncontrolled cell growth. Supernumerary centrosomes are a common characteristic of cancer cells and a source of chromosome instability. Our work provides a novel link between CENP-A posttranslational modification and the generation of chromosome instability through changes in centrosome number and cell proliferation

SECOND YEAR;

1. FASEB (Federation of American Societies for Experimental Biologist) meeting - Mitosis: Spindle Assembly and Function, Big Sky, Montana USA, June 21-26, 2015. N-terminal a-amino tri-methylation of centromere histone CENP-A required for

maintaining bipolar spindle and regulated cell proliferation. **KM Sathyan** and Daniel R. Foltz (Short talk, Invited from abstract submission)

Abstract: FASEB

N-terminal α-amino trimethylation of centromere histone CENP-A required for maintaining bipolar spindle and regulated cell proliferation

Kizhakke M. Sathyan[1], Daniel R. Foltz[1,2].

Departments of [1]Biochemistry and Molecular Genetics, and Cell Biology University of Virginia, Charlottesville, VA 22908

The deposition of histone H3 variant CENP-A marks centromere in higher eukaryotes, chromosomal loci required for accurate segregation of chromosome. It has been shown a majority of cancers exhibits chromosomal instability, however, the mechanism is unclear. Overexpression of CENP-A and its chaperone HJURP occurs in many cancers and is sufficient to cause chromosome missegregation in cell culture. The posttranslational modification (PTM) of histones resulting in the recruitment of activator and repressive complexes to chromatin is a well established and broadly used method to regulate chromatin activity by the cell. We propose that the PTMs of CENP-A have a major role in preventing chromosomal instability. Despite the identification of nearly 14 PTMs on the histone H3 amino terminus, many of which have significant functional consequences, prior to our work only a single phosphorylation on serine 7 (pSer7) of CENP-A has been described in higher eukaryotes. Recently, we have reported three new PTMs on human CENP-A N-termini using high-resolution MS: trimethylation of Gly1 and phosphorylation of Ser16 and Ser18. The nascent N-terminal residue Gly1 becomes trimethylated on the α-amino group following initial methionine removal. Here we demonstrate that the N- terminal RCC1 methyltransferase (NRMT1) methylates CENP-A both *in vitro* and *in vivo*. Even though it was identified 30 years ago, the function of N-terminal α-amino group methylation is poorly understood. Methylation occurs in the prenucleosomal as well as nucleosomal forms of CENP-A, although nucleosomal CENP-A showed higher methylation. *In vivo* as well as *in vitro* data suggest that the amino terminal tail of CENP-A is sufficient for trimethylation. We also found increase in methylation of centromeric CENP-A towards mitosis. The CENP-A methylation is required for cell survival. When CENP-A methylation mutant was replaced in a CENP-A knockout cell lines, they form significantly lower number of colonies compared to wild type replaced cells. Moreover, the CENP-A methylation resistant mutants show multipolar spindles without centriole duplication in p53 inactivated HeLa cells. A significant increase in chromosome segregation defects in HCT116 cells was also evident. The methylation mutant cells formed larger and higher number of colonies indicating uncontrolled growth in p53-/- cells, where as flatter cells in p53+/+ cells may be due to induction of senescence. When injected into mice the methylation mutant cells formed significantly more tumors than the wild type CENP-A cells. We are currently analyzing how the loss of CENP-A α-amino trimethylation leads to multipolar spindles and uncontrolled cell growth. Supernumerary centrosomes are a common characteristic of cancer cells and a source of chromosome instability. Our work

provides a novel link between CENP-A posttranslational modification and the generation of chromosome instability through changes in centrosome number and cell proliferation.

2. Chromatin, ncRNA, Methylation & Disease Symposium – NIH Bethesda, Maryland, April 16-17, 2015. α-N-methylation of CENP-A essential for chromosome segregation and cell survival. **KM Sathyan** and Daniel R. Foltz (Poster)

Abstract

N-TERMINAL α-AMINO TRIMETHYLATION OF CENTROMERE HISTONE CENP-A REQUIRED FOR MAINTAINING BIPOLAR SPINDLE AND REGULATED CELL PROLIFERATION

Sathyan, K.M.[1], Foltz, D.R.[1,2].

Departments of [1]Biochemistry and Molecular Genetics, and Cell Biology University of Virginia, Charlottesville, VA 22908

The deposition of histone H3 variant CENP-A marks centromere in higher eukaryotes, chromosomal loci required for segregation of chromosome. It has been shown a majority of cancers exhibits chromosomal instability, however, the mechanism is unclear. Overexpression of CENP-A and its chaperone HJURP occurs in many cancers and is sufficient to cause chromosome missegregation in cell culture. The posttranslational modification (PTM) of histones resulting in the recruitment of activator and repressive complexes to chromatin is a well established and broadly used method to regulate chromatin activity by the cell. We propose that the PTMs of CENP-A have a major role in preventing chromosomal instability. Despite the identification of nearly 14 PTMs on the histone H3 amino terminus, many of which have significant functional consequences, prior to our work only a single phosphorylation on serine 7 (pSer7) of CENP-A has been described in higher eukaryotes. Recently, we have reported three new PTMs on human CENP-A N-termini using high-resolution MS: trimethylation of Gly1 and phosphorylation of Ser16 and Ser18. The nascent N-terminal residue Gly1 becomes trimethylated on the α-amino group following initial methionine removal. Here we demonstrate that the N-terminal RCC1 methyltransferase (NRMT1) methylates CENP-A both *in vitro* and *in vivo*. Even though it was identified 30 years ago, the function of N-terminal α-amino group methylation is poorly understood. Methylation occurs in the prenucleosomal as well as nucleosomal forms of CENP-A, although nucleosomal CENP-A showed higher methylation. *In vivo* as well as *in vitro* data suggest that the amino terminal tail of CENP-A is sufficient for trimethylation. We also found that methylation of CENP-A increases through cell cycle with highest being found during mitosis. The CENP-A methylation is required for cell survival. When CENP-A methylation mutant was replaced in a CENP-A knockout cell lines, they form significantly lower number of colonies compared to wild type replaced cells. Moreover, the CENP-A methylation resistant mutants show multipolar spindles without centriole duplication in HeLa cells. We also found similar results in p53-/- HCT116 cells. A significant increase in chromosome segregation defects in p53-/- and p53+/+ HCT116

cells was also evident. The methylation mutant cells formed larger and higher number of colonies indicating uncontrolled growth in p53-/- cells, where as flatter cells in p53+/+ cells may be due to induction of senescence. When injected into mice the methylation mutant cells formed significantly more tumors than the wild type CENP-A cells. We are currently analyzing how the loss of CENP-A α-amino trimethylation leads to multipolar spindles and uncontrolled cell growth. Supernumerary centrosomes are a common characteristic of cancer cells and a source of chromosome instability. Our work provides a novel link between CENP-A posttranslational modification and the generation of chromosome instability.

3. Mitosis Meeting- NIH, January 22nd 2015. α-N-methylation of CENP-A essential for chromosome segregation and cell survival **KM Sathyan** and Daniel R. Foltz (talk)

4. Annual departmental retreat. Biochemistry and Molecular Genetics, University of Virginia. December 4, 2014. α-N-methylation of CENP-A essential for chromosome segregation and cell survival. **KM Sathyan** and Daniel R. Foltz (Poster)

5. Journal club. Biochemistry and Molecular Genetics, University of Virginia. November 11, 2014. α-N-methylation of CENP-A essential for chromosome segregation and cell survival (talk), **KM Sathyan.**

6.   4th Dynamic Kinetochore EMBO Workshop

Copenhagen Denmark, May 2015

Invited talk **Daniel R. Foltz**

α-AMINO TERMINAL TRIMETHYLATION OF THE CENTROMERIC HISTONE CENP-A IS REQUIRED FOR PROPER CHROMSOME SEGREGATION

Posttranslational modification (PTM) of histone amino-terminal tails is a major mechanism that regulates chromatin function. Nucleosomes containing the histone H3 variant CENP-A specify the location of the centromere, which is the locus required for segregation of chromosomes. We propose that PTMs of CENP-A have a major role in regulating centromeric chromatin and preventing chromosomal instability. We demonstrate that the methyltransferase NRMT1 (N-terminal RCC1 methyltransferase) methylates the alpha-amino group (Gly1)   of CENP-A *in vitro* and *in vivo*. Methylation occurs in the prenucleosomal as well as nucleosomal forms of CENP-A, although nucleosomal CENP-A showed higher methylation. *In vivo* as well as *in vitro* data suggest that the amino terminal tail of CENP-A is sufficient for trimethylation. Methylation of nucleosomal CENP-A increases through the cell cycle with the highest methylation level being found during mitosis. Previous work showed that the amino terminus of CENP-A is essential in human cells if the CENP-A C-terminal tail is absent. We find that CENP-A amino-terminal methylation is essential for cell survival and proper function of the CENP-A amino terminus. CENP-A methylation mutants are unable to rescue cell viability of cells in which endogenous CENP-A is knocked out. Expression of CENP-A methylation resistant mutants causes significant defects in the microtubule spindle during mitosis. We observed increased numbers of multipolar spindles in CENP-A mutant expressing cells. These multipolar spindles are not a result of centriole

duplication, suggesting that spindle defects result from an imbalance of motor forces within the mitotic spindle. Colony formation assays show that expression of CENP-A methylation mutants caused larger and higher number of colonies, demonstrating that altering CENP-A methylation may confer a proliferative advantage over wild-type CENP-A when p53 is mutated. Our work provides a novel link between CENP-A posttranslational modification and the generation of chromosome instability.

THIRD YEAR;

1. Paper Under revision:

Nature Communications

α-amino-trimethylation of CENP-A by NRMT is required full recruitment of the centromere associated network

Kizhakke M. Sathyan, Daniele Fachinetti, and Daniel R. Foltz

Abstract: Centromeres are unique chromosomal domains that control chromosome segregation and are epigenetically specified by the presence of the CENP-A containing nucleosomes. CENP-A governs centromere function by recruiting the constitutive centromere associated network (CCAN) complex. The features of the CENP-A nucleosome necessary to distinguish centromeric chromatin from general chromatin are not completely understood. Here we show that CENP-A undergoes α-amino tri-methylation by the enzyme NRMT *in vivo*. We show that α-amino methylation of the CENP-A tail contributes to cell survival. Loss of α- amino trimethylation causes a reduction in the CENP-T and CENP-I CCAN components at the centromere and leads to lagging chromosomes and spindle pole defects. The function of p53 alters the response of cells to defects associated with decreased CENP-A methylation. Together we show an important functional role for amino terminal methylation of the CENP-A nucleosome in maintaining centromere function and faithful chromosomes segregation.

2. <u>CONFERENCE</u>

1. **FASEB Biological methylation: Fundamental Mechanisms in Health and Disease,** Lisbon Portugal, June 2016

Talk selected from abstracts
α-amino trimethylation of CENP-A by NRMT is required for full recruitment of the centromere

Kizhakke M. Sathyan[1], Daniele Fachinetti[2], and **Daniel R. Foltz**

Centromeres are unique chromosomal domains that control chromosome segregation. The location of the centromere is specified by nucleosomes containing the histone H3 variant CENP-A. CENP-A governs centromere function by recruiting the constitutive centromere associated network (CCAN) complex. The features of the CENP-A nucleosome necessary to distinguish centromeric chromatin from general chromatin are

not completely understood. Posttranslational modification (PTM) of histone amino-terminal tails is a major mechanism that regulates chromatin function. We propose that PTMs of CENP-A have a major role in regulating centromeric chromatin and preventing chromosomal instability. We demonstrate that the methyltransferase NRMT1 (N-terminal RCC1 methyltransferase) methylates the alpha-amino group (Gly1) of CENP-A *in vitro* and *in vivo*. Methylation occurs in the prenucleosomal as well as nucleosomal forms of CENP-A, although nucleosomal CENP-A showed higher methylation. *In vivo* as well as *in vitro* data suggest that the amino terminal tail of CENP-A is sufficient for trimethylation. We find that CENP-A amino-terminal methylation is essential for cell survival and proper function of the CENP-A amino terminus. We show that α-amino trimethylation of the CENP-A tail contributes to cell survival. Loss of α-amino trimethylation causes a reduction in the CENP-T and CENP-I CCAN components at the centromere and leads to lagging chromosomes and spindle pole defects. The function of p53 alters the response of cells to defects associated with decreased CENP-A methylation. Together we show an important functional role for α-amino trimethylation of the CENP-A nucleosome in maintaining centromere function and chromosome stability.

Gordon Research Conference, Centromere Biology

Mount Snow VT, July 2016

"Methylation of CENP-A Is an Essential Feature of the CENP-A Amino Terminus"

Invited talk   **Daniel R. Foltz**

No abstract requested

Inventions, Patents and Licenses:
Nil

Reportable outcome:

We have got exciting results regarding the function of α-amino tri-methylation, which is under studied. We have found that the α-amino tri-methylation of CENP-A is essential for the survival of the cells and formation of the centromere. In the absence of this PTM, cells undergo senescence probably due to chromosome segregation defects caused by the improper formation of the centromere and the kinetochore. The results suggest that CENP-A α-amino tri-methylation is a crucial post-translational modification in maintaining high fidelity of chromosome segregation and any defect in this modification may results in aneuploidy and cancer. We have established that loss of CENP-A methylation in the absence of p53 has a synergistic effect in carcinogenesis. The manuscript regarding the function of α-amino tri-methylation of CENP-A is under revision in the journal Nature Communication.

Other Achievements:

Nil

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Appendices:

Abbreviations used

PTM   Post-translational modifications

CIN   Chromosomal instability

MIN	microsatellite instability
NRMT1	N-Terminal RCC1 Methyltransferase 1
NRMT2	N-Terminal RCC1 Methyltransferase 2
shRNA	short hairpin RNA
CENP-A	Centromere protein-A
CCAN	Constitutive Centromere Associated Network
α-N-me3	alpha N-terminal amino acid trimethylation
MT	Mutant
CENP-T	Centromere Protein T
CENP-I	Centromere Protein I

Opportunities for training and professional development: Last year I attended several meeting organized at the University of Virginia. I had one on one meeting with my mentor every week. We have lab meetings every other week. Moreover, we have joint meeting with Stukenberg and Burke labs. These meeting and interactions helped me in my professional development.

SECOND YEAR REPORT

Introduction
Colorectal cancer is the second leading cause of cancer death in the United States[1]. Chromosomal instability (CIN) and microsatellite instability (MIN) are two major molecular hallmarks of colorectal cancer[2,3]. 85% of colorectal cancers are CIN+ and are associated poor survival[3]. The molecular mechanisms responsible for the CIN phenotype and hence means to target this pattern of genome instability in colorectal tumors remains poorly defined. I hypothesized that, similar to the nucleosomes of general chromatin, post-translational (PTM) modifications of the centromeric nucleosome, specifically on the histone variant, CENP-A, will direct centromere activity, and that perturbations of such could lead to aneuploidy and cancer. However, the identification of CENP-A PTMs has lagged behind PTMs of other histones because of the lack of a good purification strategy. Using a novel purification strategy we have identified three PTMs on the CENP-A tail, α-amino tri-methylation of initial Glycine, and phosphorylations at S16 and S18[4]. Previously, another phosphorylation was reported at S7[5]. We proposed to decipher the pathway that leads to CENP-A α-amino methylation (CENP-A α-N-me3) and to determine the function it plays in ensuring the fidelity of chromosome segregation. We also proposed to determine how its abrogation may cause carcinogenesis and ask whether targeting this PTM is a viable strategy to target colorectal cancer cells. Overexpression of CENP-A in colorectal cancer leads to its mislocalization to chromosome arms resulting in aneuploidy. Until now the only way to inhibit CENP-A function was through shRNA knockdown. However, understanding CENP-A amino terminal tail modification will provide enzymatic and therefore potentially druggable targets to inhibit this pathway.

In the first year report we have demonstrated that CENP-A is methylated by NRMT1 both *in vitro* and *in vivo*. CENP-A is methylated before it is deposited into the centromere and that the methylation persists throughout the cell cycle. However, we found an increase in CENP-A methylation during prophase of the cell cycle. We established that CENP-A α-amino tri-methylation required for ensuring high fidelity of chromosome segregation, and hence preventing aneuploidy and cancer. Importantly, we found that loss of CENP-A α-amino tri-methylation in colorectal cancer cells trigger a proliferation advantage and they form bigger colonies in colony formation assay. Suggesting α-amino tri-methylation of CENP-A is an important post-translational modification necessary for maintaining accuracy of chromosome segregation.

This year we have made progress in further elucidating the role of CENP-A α-amino tri-methylation. Using a CENP-A knockout cell line we have found the CENP-A α-amino tri-methylation contribute to cell survival. In the absence of α-amino tri-methylation, the cells undergo senescence. CENP-A methylation required for the formation of Constitutive Centromere Associated Network (CCAN) complex. We have found in the absence of CENP-A methylation the localization of CENP-T and CENP-C is reduced. This alteration might be the reason for the chromosome segregation defect we have seen in the methylation mutant cancer cells. We inferred that the impaired formation of kinetochore might leads to an imbalance in the force generated by the motor protein on the chromosomes that leads to multipolar spindle in CENP-A methylation mutant cancer cells. Hence, we partially inhibited one of the motor proteins Eg5 using low concentration of monastrol and we were able to rescue the multipolar spindle formation. We conclude that CENP-A α-amino tri-methylation required for kinetochore formation and maintenance of bipolar spindle.

Keywords: Centromere, CENP-A, CENP-A α-amino tri-methylation, NRMT, CENP-T, CENP-C, Colorectal cancer, p53, p21

Overall project summary:
In this section we discuss our results that we accomplished during last year. Then we will discuss the importance of our finding in discussion part, and finally we will explain major materials and methods we used.

Results:
CENP-A α-amino tri-methylation required for cell survival
In order to see whether CENP-A α-amino tri-methylation is required for cell survival, we used a CENP-A knockout cell line developed by the Cleaveland lab[6]. In this cell line, one allele of the CENP-A gene is knocked out and the second allele is a Flox allele, which upon infection with adenovirus Cre-recombinase, will be removed. We have stably integrated CENP-A wild type or methylation mutant in this cell line. Since it has been shown that CENP-A n-terminal tail and c-terminal tail has some redundant function in survival of the cells[6], we replaced the c-terminal tail of CENP-A with H3 c-terminal tail in one set of mutants while keeping the n-terminus either wild type or methylation mutant (Fig.1A). The cells were infected with Ad-Cre recombinase expressing virus and after 48 hours 500 cells were seeded into 10cm plates in triplicate

(Fig.1B). After 14 days, the cells were fixed in methanol and stained with crystal violet stain. Similar to CENP-A knockout cells where only 10% of cells survived, the methylation mutant where c-terminus of the CENP-A is also replaced with H3 c-terminal tail only 10% of the cells formed colonies (Fig.1C&D). Suggesting that CENP-A α-amino tri-methylation contributes to the survival or proliferation of the cells.

Loss of CENP-A α-amino tri-methylation causes senescence

To see why the CENP-A methylation mutant did not form colonies, we checked whether the cells were undergoing senescence. We infected the wild type and mutant CENP-A cells with Ad-Cre recombinase and checked for β-galactosidase activity after 4,7 and 10 days of infection using a fluorescent substrate 5-dodecanoylaminofluorescein di-β-D-galactopyranoside (C 12FDG) (Fig.2A&B)[7]. The CENP-A knockout cell line without any CENP-A replacement upon infection with Ad-Cre virus underwent senescence (Fig.2C). However, cells replaced with CENP-A wild type or full-length methylation mutant did not show senescence phenotype. In this case we found only less that 1% cells undergoing senescence compared to 20% in CENP-A knockout cell line. However, the methylation mutant where the c-terminal tail changed to H3 c-terminus underwent senescence at a rate similar to the CENP-A knockout cell lines (Fig.2D). This suggests that in the absence of CENP-A α-amino tri-methylation, the n-terminal tail is no longer fully functional and could not compensate for the loss of c-terminal tail. Suggesting the CENP-A α-amino tri-methylation contributes to the survival of the cells and its loss leads to senescence in normal cells.

We checked the senescence induction is through p53[8]. We found that upon endogenous CENP-A removal, the p53 level is elevated and is reduced in CENP-A wild type replaced cells (Fig.2E). But in the methylation mutant where the c-terminal tail changed to H3 c-terminus we see an overall increase in p53 even without removing the endogenous CENP-A and is further induced upon endogenous CENP-A removal (Fig.2E). This increased p53 level even in the presence of endogenous CENP-A renders the cell susceptible to senescence.

Figure 1

A

me3-CENP-A-GFP

MT-CENP-A-GFP

me3-CENP-A –C-H3-GFP

MT–CENP-A –C-H3-GFP

Viral integration

B

Mutant or Wild-type
CENP-A-GFP
Rescue

Plate and grow
for 14 days

Ad-Cre
infection

CENP-A $^{-/Flox}$

CENP-A $^{-/Flox}$
CENP-A-GFP

CENP-A $^{-/-}$
CENP-A-GFP

C

Untreated   Ad-Cre

CENP-A $^{-/Flox}$

me3-CENP-A-GFP

MT-CENP-A-GFP

me3-CENP-A-C-H3-GFP

MT-CENP-A-C-H3-GFP
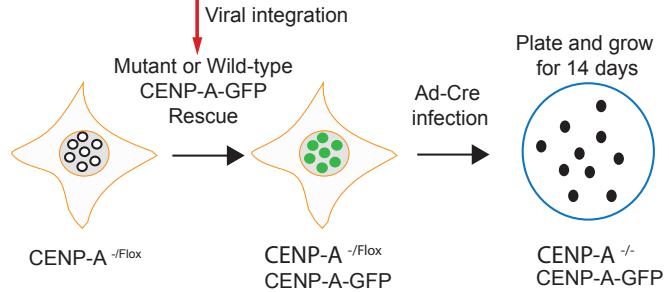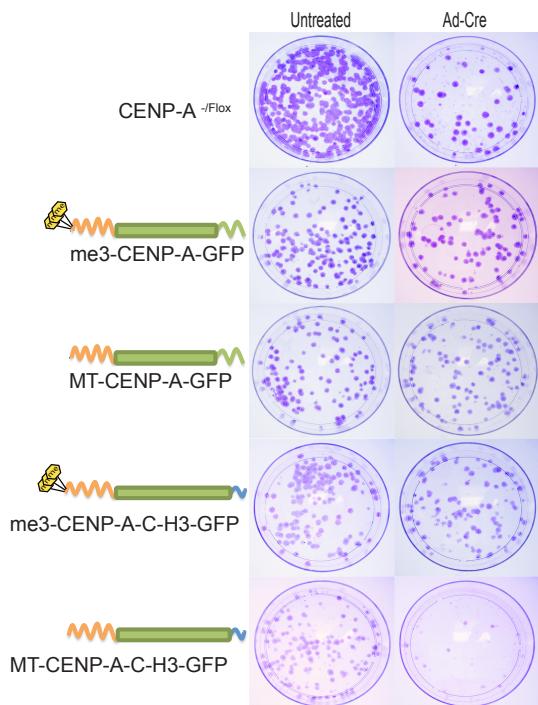
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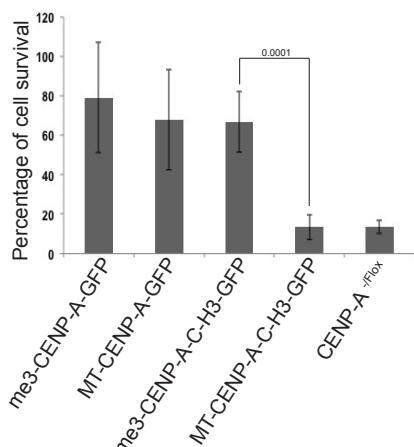
Figure 1. CENP-A α-amino tri-methylation required for cell survival (A). Schematic diagram of the different CENP-A constructs used, (B). Schematics of the experiment. RPE CENP-A knockout cell line RPE CENP-A -/F cells were virally integrated with CENP-A constructs. The second allele of the endogenous CENP-A was then removed by infecting with Ad-cre virus. 48 hours after infection, the cells were split and 500 cells were seeded onto 10cm plate in triplicate. After 14 days the colonies were visualized by fixing in methanol and crystal violet staining, (C). Representative images of the colony formation assay. The number of colonies formed drastically reduced upon CENP-A removal that is restored with all the wild type and mutant CENP-A except the methylation mutant where c-terminus is also swapped with H3 c-terminus, (E). Graph showing percentage of cell survival calculated based on colony formation assay.

The same is true for p21 level (Fig.2E). Suggesting that loss of CENP-A/CENP-A α-amino tri-methylation induce senescence in a p53 dependent manner.

Figure 2
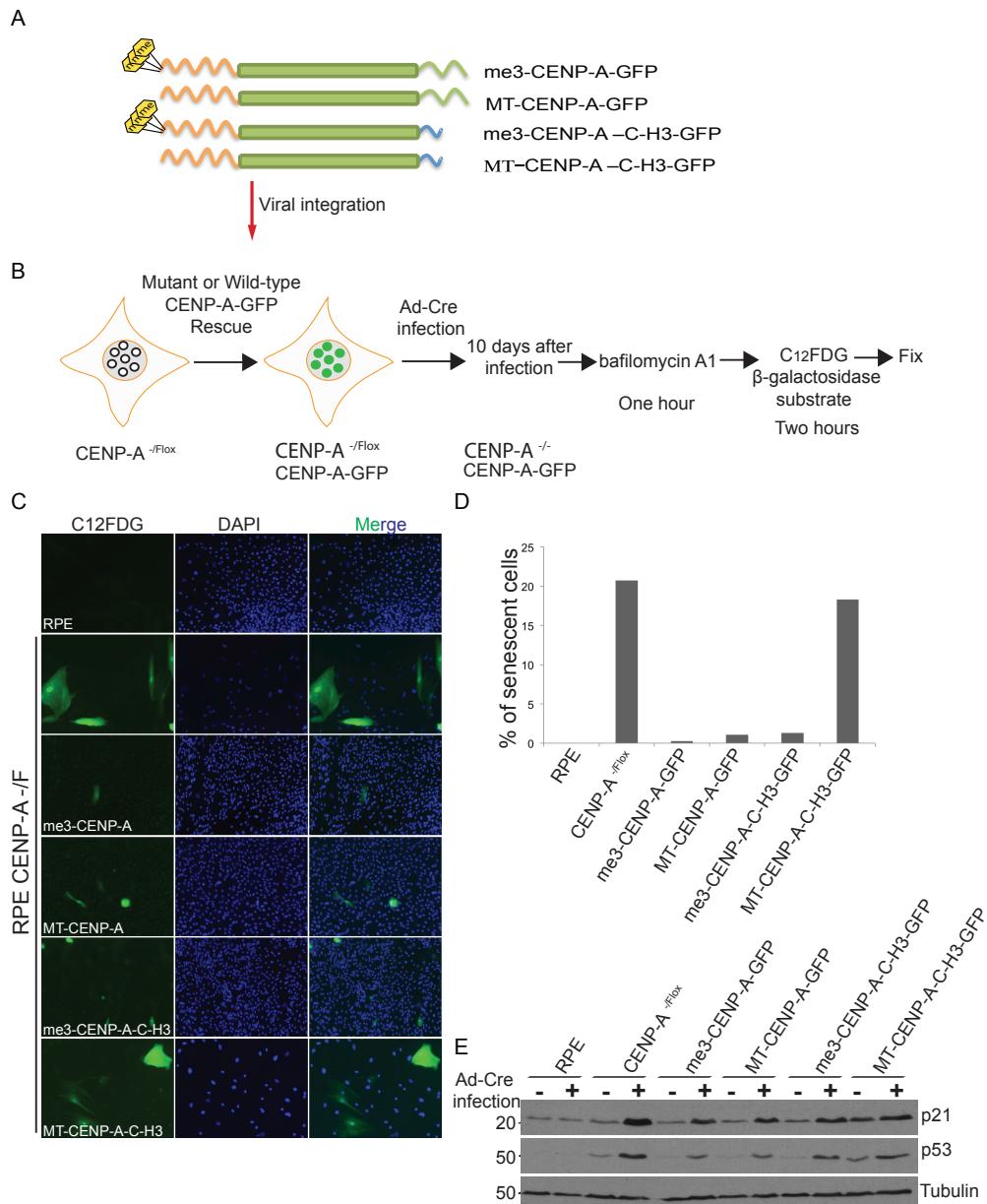
Figure 2. Loss of CENP-A α -amino tri-methylation causes senescence (A). Schematic diagram of the different CENP-A constructs used, (B). Schematics of the experiment. CENP-A knockout cell line RPE CENP-A -/F cells were virally integrated with CENP-A constructs. The second allele of the endogenous CENP-A was then removed by infecting with Ad-cre virus. On days 4, 7 and 10, the cells were treated with Bafilomycin A1 for one hour and then added 5-dodecanoylaminofluorescein di-β-D-galactopyranoside (C$_{12}$FDG), a fluorogenic substrate for β-galactosidase for another hour. The cells were then fixed, permeabilized and DAPI stained. The cells were observed under microscope. A green fluorescent staining indicates senescent cells, (C). Representative images of the β-galactosidase assay. Upon CENP-A removal the cells undergo senescence, which is rescued by all the CENP-A, constructs used except CENP-A methylation mutant where c-terminal of CENP-A is also swapped with H3 c-terminus (lower panel), (D). Graph showing percentage of senescent cells, (E). Western blot showing p53 and p21 induction

after CENP-A knockout. P53 and p21 induced upon removal of endogenous CENP-A which is rescued partially by exogenous wild type and mutant CENP-A except CENP-A methylation mutant where c-terminal of CENP-A is also swapped with H3 c-terminus. p53 and p21 remained high in this mutant cells even in the presence of endogenous CENP-A, which is further induced upon endogenous CENP-A removal by infecting with Ad-cre virus.

α-amino tri-methylation of CENP-A required for the accurate formation of CCAN complex

Since CENP-A epigenetically specifies centromere and involved in the assembly of Constitutively Centromere Associated Network (CCAN) complex, we wanted to check whether loss of CENP-A methylation cause defects in CCAN recruitment at the centromere. For this we used the CENP-A knockout RPE cells where we stably integrated either wild type or mutant CENP-A (Fig.3A). The localization of CCAN proteins such as CENP-C, CENP-T, CENP-I and CENP-B where analyzed with and without Ad-Cre virus infection which removes endogenous CENP-A. In our initial analysis, we found a consistent reduction in CENP-T (Fig.3E&F) and CENP-C (Fig.3E&G) in CENP-A methylation mutant compared to wild type replaced cells similar to CENP-A knockout cell line. However, we did not find such a difference in the localization of these proteins where endogenous CENP-A is still present (Fig.3B,C&D). This suggests that CENP-A α-amino tri-methylation is required for the accurate assembly of CCAN components. This result may explain the chromosome segregation defects and multipolar spindle formation that we see in the CENP-A methylation mutant cancer cells (Last year report). Thus, CENP-A methylation is required for the formation of the proper centromere formation and efficient chromosome segregation.

Force imbalance generated by the lagging chromosomes causes multipolar spindle in CENP-A methylation mutant

It has been shown that force balance generated by different motor proteins such as Kif15, Eg5, CENP-E and Dynein is required for the formation of bipolar spindle[9]. Alterations in the centromere and kinetochore structure could lead to improper localization of these factors. To see whether the multipolarity is caused by the force imbalance generated by the unaligned chromosomes, we partially inhibited the motor protein Eg5. CENP-A was knockdown in HeLa cells using shRNA as depicted in the diagram (Fig.4A) and synchronized using a double Thymidine block and release. Twelve hours after release we added either MG132 or MG132 along with Eg5 inhibitor monastrol. We found that percentage of cells with multipolar spindles was reduced to control levels in CENP-A shRNA treated cells after treatment with Monastrol. This suggests that a force imbalance generated by the improper kinetochore formation after knockdown of CENP-A is the reason for the formation of multipolar spindle (Fig.4B&C). To see whether a similar force imbalance causes multipolar spindles in CENP-A methylation mutants, we knockdown and replaced CENP-A wild type or mutant CENP-A. In these cells we treated cells with MG132 along with increasing concentration of monastrol. With increase in monastrol concentration we observed a decrease in multipolar spindle in CENP-A methylation mutant (Fig.4D). This suggests that in CENP-A methylation mutant force imbalance generated by the defective chromosome segregation causes multipolarity. We were also seen similar effects in HCT116 colorectal cancer cells.
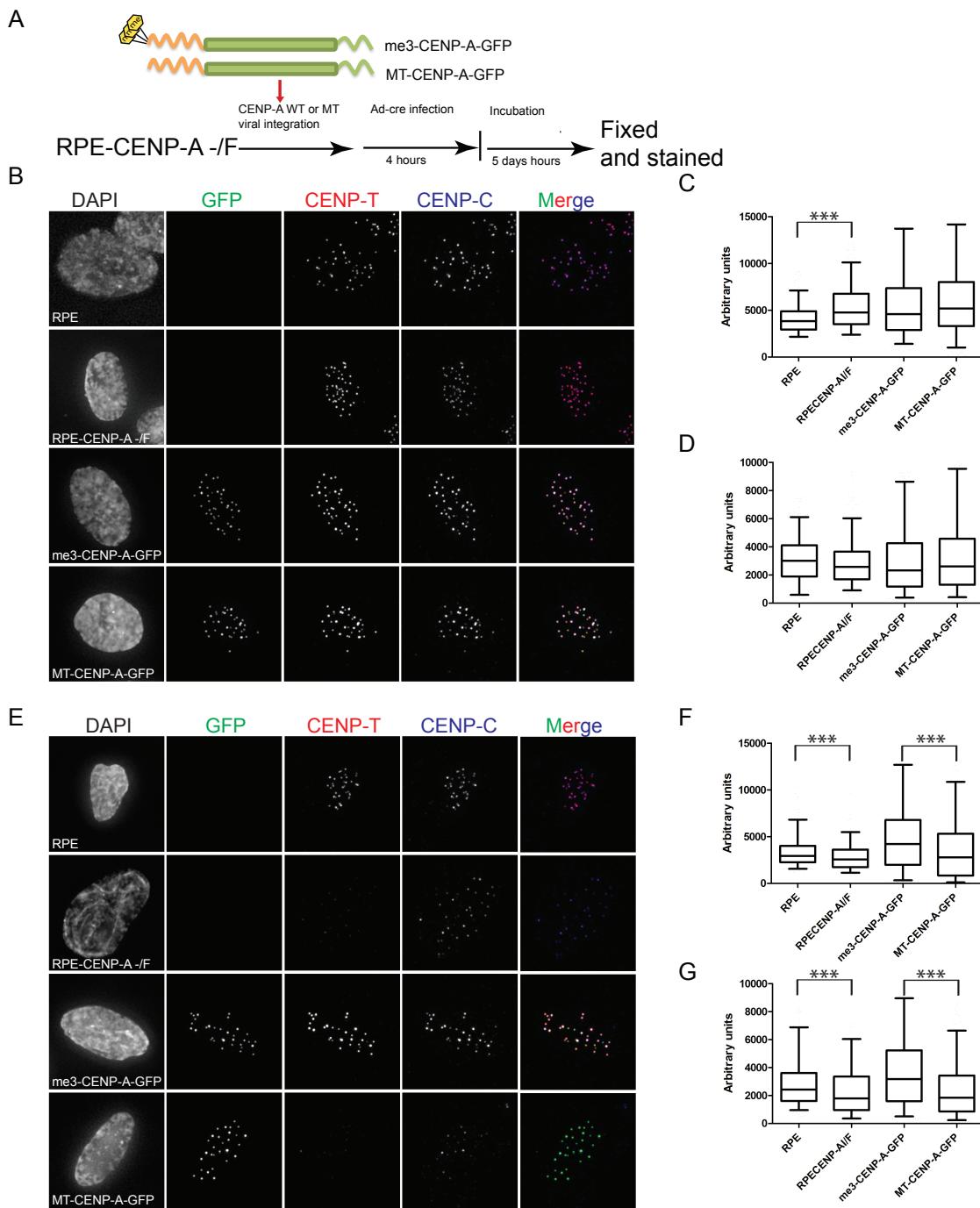
Figure 3. α-amino tri-methylation of CENP-A required for the accurate formation of CCAN complex (A). Schematic diagram of the experiment. CENP-A knockout cell line RPE CENP-A -/F cells were virally integrated with full-length wild type or mutant CENP-A constructs. The endogenous CENP-A was then removed by Ad-cre virus infection. On 5th day the cells were pre-extracted, fixed and stained with CENP-T and CENP-C antibodies, (B). CENP-T and CENP-C staining before Ad-cre infection. The rescue construct shown as green (GFP), (C&D). Quantitation of CENP-T and CENP-C at the centromere before infecting with Ad-cre virus respectively, (E) CENP-T and CENP-C centromere localization after the removal of endogenous CENP-A by Ad-cre virus, (F&G). Quantitation of CENP-T and CENP-C at the centromere respectively after the removal of endogenous CENP-A by Ad-cre virus. *** p value <0.0001.

Figure 4

A

B

C

CENP-A shRNA	-	+	+	+
Mg132	-	-	+	+
Monastrol	-	-	-	+

D

CENP-A shRNA	+	+	+	+	+	+
Mg132	-	+	+	+	+	-
Monastrol	-	-	7.5	15	22.5	DMSO
CENP-A replacement	WT	MT	MT	MT	MT	MT
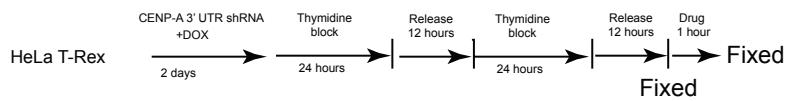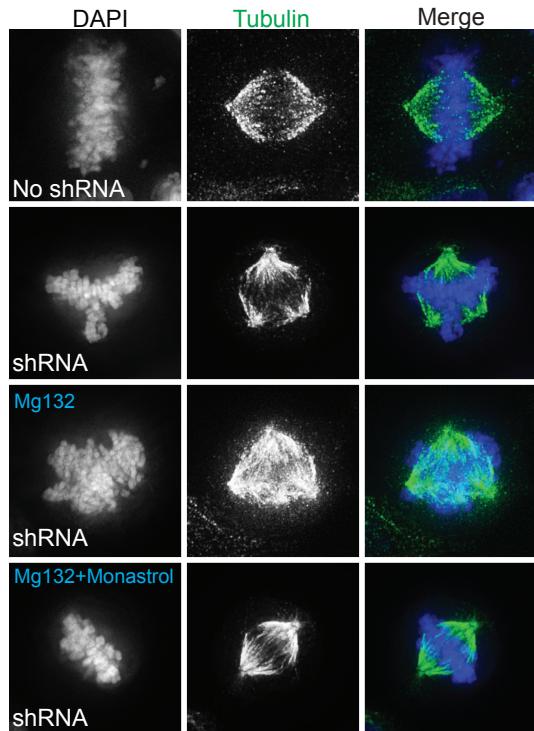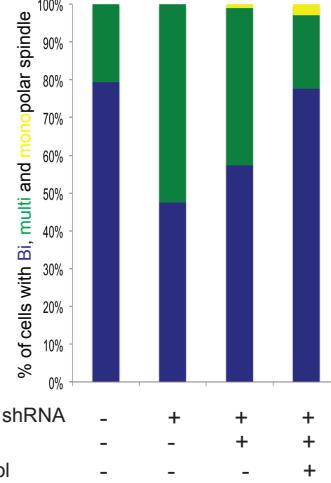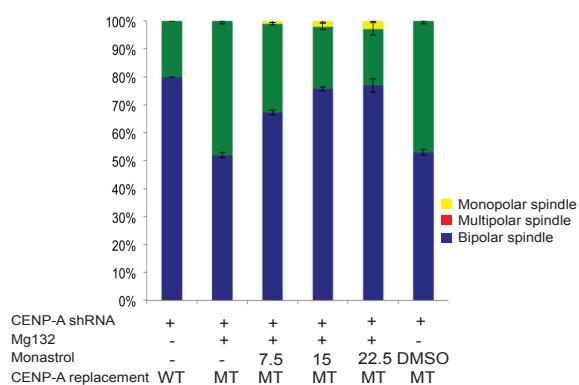
Figure 4. Force imbalance generated by the misaligned chromosomes causes multipolar spindle in CENP-A methylation mutant (A). Schematic diagram of the experiment. Endogenous CENP-A was knocked down either with or without replacement for eight days. The cells then double thymidine blocked and released. 12 hours after second release cells were treated with indicated drugs for one hour and cells were fixed and stained, (B). Mitotic cells stained for tubulin after with and without CENP-A knock down and treated with Eg5 inhibitor monastrol and proteasome inhibitor MG132. Treatment with monastrol decreases the multipolar spindle formation, (C&&D). Percentages of the monopolar, bipolar and multipolar cells with CENP-A knockdown (C) and replacement cells (D) treated with monastrol.

Discussion:

The post-translational methylation of alpha-amino groups was first discovered over 30 years ago[10,11]. However, its biological function remains obscure except in the case RCC1 (Ran guanine nucleotide-exchange factor) where it is required for its localization into chromosomes[11]. We identified a novel function of alpha-N-methylation. We found that alpha-N-methylation of CENP-A is required for maintaining genomic stability and its loss leads to aneuploidy. We demonstrated that CENP-A is methylated throughout the cell cycle and methylated CENP-A increases during prophase. Reduction in CENP-A level increases the propensity of multipolar spindle pole formation and lagging chromosome in p53 null colorectal cancer cell HCT116. Where as in isogenic p53+/+ cells there is no significant increase in multipolar spindle formation after CENP-A knockdown. In our knockdown replacement experiment, wild type CENP-A stably integrated cells had similar percentage of multipolar cells as that of parental cell lines both in colorectal cancer cell HCT116 p53-/- and HeLa. But the methylation mutant CENP-A stably integrated cells showed significantly increased multipolar spindle in p53-/- HCT116 and p53 inactive HeLa cells suggesting CENP-A methylation mutants are hypomorphic. We also analyzed chromosome missegregation defects in these cells and found that there is an increase in lagging chromosomes compared to wild type irrespective of p53 status. We also found further evidence that CENP-A methylation has a protective effect in tumorigenesis.

Surprisingly, here we demonstrated that CENP-A methylation contributes to the survival and/or proliferation of the immortalized RPE cells. CENP-A's n-terminal and c-terminal tails have some redundant function in cell survival[6]. In the absence of c-terminal tail, the CENP-A methylation mutant could not form colonies, suggesting the n-terminal is non functional without methylation. We also found that loss of CENP-A methylation in CENP-A without c-terminal tail cells trigger a senescence response. CENP-A knockout cell lines also undergo senescence in a p53 dependent manner. In the methylation mutant CENP-A, we found an overall increase in p53. Suggesting that cells sensing a functional CENP-A in the cells and in the absence of functional CENP-A it induce senescence to reduce aneuploidy. But in the absence of p53, cells accumulate chromosome segregation defects and become more tumorigenic.

Our preliminary results showed that there is a reduction in the centromere localization of CCAN components in CENP-A methylation mutant cells. The centromere localization of CENP-C and CENP-T is significantly reduced in methylation mutant cells. This suggests that CENP-A methylation is partially involved in the localization of these proteins to the centromere. Such changes in CCAN might be the reason for the chromosome segregation defects and multipolar spindle in the CENP-A methylation mutant cancer cells due to defective kinetochore formation.

Since we have seen chromosome segregation defects and multipolar spindle formation in CENP-A methylation mutant in cancer cells, we hypothesize that force imbalance generated by different motor proteins may be the reason for multipolar spindle formation. In such cases, we will see a reduction in multipolarity if we partially inhibit such motor proteins. Upon partial inhibitions of Eg5 with low concentrations of monastrol at which concentration it does not induce monopolar spindle, we found that multipolarity reduced to the control level. Suggesting that indeed force imbalance generated by the improper formation of kinetochore is the mechanism for the formation of multipolar spindle.

Overall we have discovered that CENP-A methylation contributes to cell survival and in the absence of methylation cells undergo senescence. However, in the absence of p53, loss of CENP-A methylation causes multipolar spindle and confer more tumorigenic potential to the cancer cells. We still need to further confirm the CCAN localization defects in CENP-A methylation mutant and the role of CENP-A methylation in cancer,[12] for that a continuous support is necessary.

Materials and methods:

Colony formation assay: RPE CENP-A knockout cell line generated by the Cleaveland Lab[6] used for this study. In this cell lines one allele of CENP-A is knocked out and the other allele is a Flox allele, which upon Ad-cre recombinase virus infection will be removed. We made CENP-A wild type and mutant stable lines in these cells. 2500 cells were infected with Ad-cre for four hours and then the virus was washed out. 48 hours after infection, 500 cells were split into 10cm plates. The colonies were crystal violet stained after 14 days and counted.

Senescence assay: RPE CENP-A knockout cell lines integrated with either wild type or mutant CENP-A was infected with Ad-cre virus as mentioned above. After 4,7 and 10 days cells were treated with Bafilomycin-A1 for one hour and then added 5-dodecanoylaminofluorescein di-β-D-galactopyranoside (C_{12}FDG), a fluorogenic substrate for b-galactosidase. Cells were fixed after one hour and permeabilized with 0.1% triton in PBS. The nucleus was stained with DAPI and mounted.

Monastrol treatment: CENP-A shRNA integrated into HeLa T-Rex cells where induced by adding doxycycline for seven days. The cells were then double thymidine block and release. 12 hours after second release cells were either directly fixed or treated one hour with MG132 or MG132 with low concentration of monastrol. Cells were fixed and stained for tubulin. Similarly, in the knockdown replacement experiment, endogenous CENP-A was knockdown in wild type or mutant replaced cells. The cells were double thymidine blocked and released. 12 hours after second release cells were either directly fixed or treated one hour with MG132 or MG132 with increasing concentration of monastrol. Cells were then fixed and stained for tubulin. The multipolar cells were counted.

Immunofluorescence: Cells were either pre-extracted with 0.1% triton in 1X PBS for 3 minutes and then fixed in 4% formaldehyde for 10 minutes. The cells were then blocked in 2% BSA and 2% fetal calf serum with 0.1% triton in 1XPBS. It was incubated with primary antibodies one hour and then secondary antibody was added for one hour. All washes between each step were done in 1XPBS + 0.1% triton. DNA was stained with DAPI and were mounted in prolong. Cells were examined and images were acquired using a Delta Vision optical sectioning deconvolution instrument (Applied Precision) using a X100 oil-immersion Olympus objective lens connected with Photometrics CoolSNAP HQ^2 camera and Softwrox acquisition software. Images were deconvolved and presented as maximum stacked images. The antibodies used for immunofluorescence were: mouse anti-CENP-A (1:1000), Rabbit anti-me3CENP-A (1:200), Rabbit anti-CENP-T (1:1000), Mouse anti-CENP-C (1:1000), Rabbit anti-CENP-I (1:1000, Mouse anti-CENP-B (1:250), Mouse anti-α-tubulin (1:1000).

Following antibodies were used for western blotting. Mouse anti-p53 (DO7, Santa Cruz) (1:1000), Rabbit anti-p21(Santa Cruz) (1:500), Mouse anti-tubulin (1:1000).

Key accomplishments:

Following are the key accomplishments of the proposed work

Year I

8. Identified NRMT1 methylates CENP-A at its α-amino terminal.
9. CENP-A is methylated throughout the cell cycle.
10. Pre-nucleaosomal CENP-A is methylated.
11. We made several methylation resistant CENP-A mutants.
12. Loss of CENP-A cause aneuploidy by forming multipolar spindle and missegregation of chromosomes.
13. α-amino tri-methylation of CENP-A ensures high fidelity of chromosome segregation. Its loss cause multipolar spindle and lagging chromosomes in p53 null background and lagging chromosomes in the presence of p53. Both these alterations fundamentally cause aneuploidy, a form of genetic alterations prevalent (85%) in colorectal cancer.
14. We also found that methylation mutant CENP-A forms bigger colonies in colony formation assay suggesting role of methylation in controlling proliferation.

Year II
6. CENP-A α-amino tri-methylation required for the survival of the cells.
7. Loss of α-amino tri-methylation of CENP-A leads to senescence in normal cells.
8. Loss of CENP-A induces p53 and that leads to senescence. This explains the increased tumorigenic potential seen in CENP-A methylation mutant, which is less functional, in p53 null cells.
9. α-amino tri-methylation of CENP-A required for the localization of CCAN components to the centromere and centromere homeostasis.
10. Force imbalance generated by the improper kinetochore formation and resultant misaligned chromosomes may be the reason for the multipolar spindle formation in p53 null CENP-A mutant cell lines.

My abstract was selected for a short talk in the prestigious FASEB (Federation of American Societies for Experimental Biologist) meeting - Mitosis: Spindle Assembly and Function. Moreover I gave talk and did poster presentation in several meetings within the University and outside. A list of the talk and poster presentation that I gave during the last year is given in the publication, abstract and presentation part.

Conclusion:

We have made significant progress in elucidating the function of α-amino tri-methylation of CENP-A. We achieved several of the proposed aims. We found that CENP-A is methylated by NRMT1 and this modification persists throughout the cell cycle. We also found that α-amino tri-methylation of CENP-A is critical in orchestrating chromosome segregation and its abrogation may leads to aneuploidy and cancer. We also found that this modification required for the survival of the cells and in the absence of this modification, normal cells undergo senescence. We were able to show that α-amino tri-methylation of CENP-A is required for the efficient recruitment of CCAN proteins at the centromere. The accurate formation of CCAN and kinetochore is essential for high fidelity chromosome segregation. We have made significant progress in the proposed study but further support is necessary to fully understand the function of α-amino tri-methylation of CENP-A and its role in colorectal cancer.

Publications, Abstracts, and Presentations:

1. FASEB (Federation of American Societies for Experimental Biologist) meeting - Mitosis: Spindle Assembly and Function, Big Sky, Montana USA, June 21-26, 2015. N-terminal a-amino tri-methylation of centromere histone CENP-A required for maintaining bipolar spindle and regulated cell proliferation. KM Sathyan and Daniel R. Foltz (Short talk)

Abstract: FASEB

N-terminal α-amino trimethylation of centromere histone CENP-A required for maintaining bipolar spindle and regulated cell proliferation

Kizhakke M. Sathyan[1], Daniel R. Foltz[1,2].

Departments of [1]Biochemistry and Molecular Genetics, and Cell Biology University of Virginia, Charlottesville, VA 22908

The deposition of histone H3 variant CENP-A marks centromere in higher eukaryotes, chromosomal loci required for accurate segregation of chromosome. It has been shown a majority of cancers exhibits chromosomal instability, however, the mechanism is unclear. Overexpression of CENP-A and its chaperone HJURP occurs in many cancers and is sufficient to cause chromosome missegregation in cell culture. The posttranslational modification (PTM) of histones resulting in the recruitment of activator and repressive complexes to chromatin is a well established and broadly used method to regulate chromatin activity by the cell. We propose that the PTMs of CENP-A have a major role in preventing chromosomal instability. Despite the identification of nearly 14 PTMs on the histone H3 amino terminus, many of which have significant functional consequences, prior to our work only a single phosphorylation on serine 7 (pSer7) of CENP-A has been described in higher eukaryotes. Recently, we have reported three new PTMs on human CENP-A N-termini using high-resolution MS: trimethylation of Gly1 and phosphorylation of Ser16 and Ser18. The nascent N-terminal residue Gly1 becomes trimethylated on the α-amino group following initial methionine removal. Here

we demonstrate that the N- terminal RCC1 methyltransferase (NRMT1) methylates CENP-A both *in vitro* and *in vivo*. Even though it was identified 30 years ago, the function of N-terminal α-amino group methylation is poorly understood. Methylation occurs in the prenucleosomal as well as nucleosomal forms of CENP-A, although nucleosomal CENP-A showed higher methylation. *In vivo* as well as *in vitro* data suggest that the amino terminal tail of CENP-A is sufficient for trimethylation. We also found increase in methylation of centromeric CENP-A towards mitosis. The CENP-A methylation is required for cell survival. When CENP-A methylation mutant was replaced in a CENP-A knockout cell lines, they form significantly lower number of colonies compared to wild type replaced cells. Moreover, the CENP-A methylation resistant mutants show multipolar spindles without centriole duplication in p53 inactivated HeLa cells. A significant increase in chromosome segregation defects in HCT116 cells was also evident. The methylation mutant cells formed larger and higher number of colonies indicating uncontrolled growth in p53-/- cells, where as flatter cells in p53+/+ cells may be due to induction of senescence. When injected into mice the methylation mutant cells formed significantly more tumors than the wild type CENP-A cells. We are currently analyzing how the loss of CENP-A α-amino trimethylation leads to multipolar spindles and uncontrolled cell growth. Supernumerary centrosomes are a common characteristic of cancer cells and a source of chromosome instability. Our work provides a novel link between CENP-A posttranslational modification and the generation of chromosome instability through changes in centrosome number and cell proliferation.

2. Chromatin, ncRNA, Methylation & Disease Symposium – NIH Bethesda, Maryland, April 16-17, 2015. α-N-methylation of CENP-A essential for chromosome segregation and cell survival. KM Sathyan and Daniel R. Foltz (Poster)

Abstract: NIH

N-TERMINAL α-AMINO TRIMETHYLATION OF CENTROMERE HISTONE CENP-A REQUIRED FOR MAINTAINING BIPOLAR SPINDLE AND REGULATED CELL PROLIFERATION

Sathyan, K.M.[1], Foltz, D.R.[1,2].

Departments of [1]Biochemistry and Molecular Genetics, and Cell Biology University of Virginia, Charlottesville, VA 22908

The deposition of histone H3 variant CENP-A marks centromere in higher eukaryotes, chromosomal loci required for segregation of chromosome. It has been shown a majority of cancers exhibits chromosomal instability, however, the mechanism is unclear. Overexpression of CENP-A and its chaperone HJURP occurs in many cancers and is sufficient to cause chromosome missegregation in cell culture. The posttranslational modification (PTM) of histones resulting in the recruitment of activator and repressive complexes to chromatin is a well established and broadly used method to regulate chromatin activity by the cell. We propose that the PTMs of CENP-A have a major role in preventing chromosomal instability. Despite the identification of nearly 14

PTMs on the histone H3 amino terminus, many of which have significant functional consequences, prior to our work only a single phosphorylation on serine 7 (pSer7) of CENP-A has been described in higher eukaryotes. Recently, we have reported three new PTMs on human CENP-A N-termini using high-resolution MS: trimethylation of Gly1 and phosphorylation of Ser16 and Ser18. The nascent N-terminal residue Gly1 becomes trimethylated on the α-amino group following initial methionine removal. Here we demonstrate that the N-terminal RCC1 methyltransferase (NRMT1) methylates CENP-A both *in vitro* and *in vivo*. Even though it was identified 30 years ago, the function of N-terminal α-amino group methylation is poorly understood. Methylation occurs in the prenucleosomal as well as nucleosomal forms of CENP-A, although nucleosomal CENP-A showed higher methylation. *In vivo* as well as *in vitro* data suggest that the amino terminal tail of CENP-A is sufficient for trimethylation. We also found that methylation of CENP-A increases through cell cycle with highest being found during mitosis. The CENP-A methylation is required for cell survival. When CENP-A methylation mutant was replaced in a CENP-A knockout cell lines, they form significantly lower number of colonies compared to wild type replaced cells. Moreover, the CENP-A methylation resistant mutants show multipolar spindles without centriole duplication in HeLa cells. We also found similar results in p53-/- HCT116 cells. A significant increase in chromosome segregation defects in p53-/- and p53+/+ HCT116 cells was also evident. The methylation mutant cells formed larger and higher number of colonies indicating uncontrolled growth in p53-/- cells, where as flatter cells in p53+/+ cells may be due to induction of senescence. When injected into mice the methylation mutant cells formed significantly more tumors than the wild type CENP-A cells. We are currently analyzing how the loss of CENP-A α-amino trimethylation leads to multipolar spindles and uncontrolled cell growth. Supernumerary centrosomes are a common characteristic of cancer cells and a source of chromosome instability. Our work provides a novel link between CENP-A posttranslational modification and the generation of chromosome instability.

3. Mitosis Meeting- NIH, January 22nd 2015. α-N-methylation of CENP-A essential for chromosome segregation and cell survival KM Sathyan and Daniel R. Foltz (talk)

4. Annual departmental retreat. Biochemistry and Molecular Genetics, University of Virginia. December 4, 2014. α-N-methylation of CENP-A essential for chromosome segregation and cell survival. KM Sathyan and Daniel R. Foltz (Poster)

5. Journal club. Biochemistry and Molecular Genetics, University of Virginia. November 11, 2014. α-N-methylation of CENP-A essential for chromosome segregation and cell survival (talk)

Inventions, Patents and Licenses:
Nil

Reportable outcome:

We have got exciting results regarding the function of α-amino tri-methylation, which is under studied. We have found that the α-amino tri-methylation of CENP-A is essential for the survival of the cells and formation of the centromere. In the absence of this PTM, cells undergo senescence probably due to chromosome segregation defects caused by the improper formation of the centromere and the kinetochore. The results suggest that CENP-A α-amino tri-methylation is a crucial post-translational modification in maintaining high fidelity of chromosome segregation and any defect in this modification may results in aneuploidy and cancer. We are writing a manuscript regarding the function of α-amino tri-methylation of CENP-A, which will be submitted in the Nature Cell Biology Journal.

Other Achievements:

Nil

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Appendices:

Abbreviations used

PTM	Post-translational modifications
CIN	Chromosomal instability
MIN	microsatellite instability
NRMT1	N-Terminal RCC1 Methyltransferase 1
NRMT2	N-Terminal RCC1 Methyltransferase 2
shRNA	short hairpin RNA
CENP-A	Centromere protein-A
CCAN	Constitutive Centromere Associated Network
α-N-me3	alpha N-terminal amino acid trimethylation
MT	Mutant

Opportunities for training and professional development: Last year I had several opportunities for my professional development. I got an opportunity to give a short talk in the prestigious FASEB meeting - Mitosis: Spindle Assembly and Function. I also presented a poster in a NIH organized meeting. Moreover Foltz lab had joint meeting with six different labs who are working in the mitosis field. The meeting was held in NIH. I gave a talk about my work in that meeting. I also had several opportunities to present my work in various meetings in the University of Virginia.

I meet my mentor every week and discuss research progress. We have lab meetings

every other week. Moreover, we have joint meeting with Stukenberg and Burke labs every week. These meeting and interactions helped me in my professional development.

FIRST YEAR REPORT

Introduction

Colorectal cancer is the second leading cause of cancer death in the United States[1]. Chromosomal instability (CIN) and microsatellite instability (MIN) are two major molecular hallmarks of colorectal cancer[2,3]. 85% of colorectal cancers are CIN+ and are associated with poor survival[3]. The molecular mechanisms responsible for the CIN phenotype and hence means to target this pattern of genome instability in colorectal tumors remains poorly defined. I hypothesize that, similar to the nucleosomes of general chromatin, post-translational (PTM) modifications of the centromeric nucleosome, specifically on the histone variant, CENP-A, will direct centromere activity, and that perturbations of such could lead to aneuploidy and cancer. However, the identification of CENP-A PTMs has lagged behind PTMs of other histones because of the lack of a good purification strategy. Using a novel purification strategy we have identified three PTMs on the CENP-A tail, α-amino tri-methylation of initial Glycine, and phosphorylations at S16 and S18. Previously, another phosphorylation was reported at Serine S7[4]. In our original application, we proposed to decipher the pathway that leads to CENP-A α-amino methylation (CENP-A α-N-me3) and to determine the function it plays in ensuring the fidelity of chromosome segregation. We also proposed to determine how its abrogation may cause carcinogenesis and ask whether targeting this PTM is a viable strategy to target colorectal cancer cells. Overexpression of CENP-A in colorectal cancer leads to its mislocalization to chromosome arms resulting in aneuploidy. Until now the only way to inhibit CENP-A function was through shRNA knockdown. However, understanding CENP-A amino terminal tail modification will provide enzymatic and therefore potentially druggable targets to inhibit this pathway.

We have shown here that CENP-A is methylated by NRMT1 both *in vitro* and *in vivo*. CENP-A is methylated before it is deposited into the centromere and that the methylation persists throughout the cell cycle. However, we found an increase in CENP-A methylation during prophase of the cell cycle, an observation that merits further studies. We established that CENP-A α-amino tri-methylation is required for ensuring high fidelity of chromosome segregation, and hence preventing aneuploidy and cancer. Importantly, we found that loss of CENP-A α-amino tri-methylation in colorectal cancer cells results in bigger colonies in colony formation assay suggesting that this lesion triggers a proliferative advantage. Our new data support the hypothesis that α-amino tri-methylation of CENP-A is an important post-translational modification necessary for maintaining accuracy of chromosome segregation.

Body

In this section we discuss our results we have accomplished during the first year of this DOD award. Then we will discuss the importance of our finding in discussion section, and finally we will explain major materials and methods we used.

Results:
NRMT Methylates CENP-A

As part of the original proposal, we raised an antibody against amino-terminally trimethylated CENP-A (me3-CENP-A), in order to readily assess CENP-A methylation *in vivo.* We used a peptide blocking experiment to reveal that the antibody is specific. In this experiment, only the antibody blocked with me3-CENP-A peptide showed loss of centromeric staining indicating it is specific to methylated CENP-A (included in the proposal) (Fig.1A). We also depleted CENP-A using shRNA and immunostained for me3-CENP-A. The results show that CENP-A knockdown completely abolished centromeric staining (Fig.1B). Both these results indicating that the newly raised antibody recognize methylated CENP-A.

Our previous results show that CENP-A N-terminal methionine is cleaved *in vivo* (Fig.1C)[5]. So far, four post-translational modifications on CENP-A have been discovered, alpha-N-methylation of Glycine 1, and phosphorylations at Serine S7, S16 and S18[4,5]. The majority of CENP-A is tri-methylated at the glycine alpha-amino group after methionine removal[5] (Fig.1C). There are two mammalian enzymes that methylate proteins at their alpha amino terminal, NRMT1 and 2. NRMT1 tri-methylates proteins where as NRMT2 is a mono-methylase[6,7]. We purified His-tagged human NRMT1 from bacteria and did an *in vitro* methylation assay. To obtain CENP-A N-terminus devoid of methionine, we expressed a CENP-A fusion protein where a 6XHis tag followed by Factor X protease cleavage site and amino acids 2-10 of CENP-A initial 10 amino acids were fused to the N-terminus of GFP (Fig1.D). The expressed fusion was then purified with nickel beads (Fig.1D), cleaved with Factor X, and negatively selected with nickel beads (Fig.1D). This exposes the glycine 1, which is the first amino acid of CENP-A N-terminus after methionine. This exposed CENP-A amino terminal fusion protein was used as substrate in an *in vitro* NRMT methylation analysis. The proteins then run on a gel and western blotted for GFP and me3-CENP-A antibody (Fig.1E, also shown in the proposal). The me3-CENP-A antibody recognized a band only in reactions having NRMT, clearly showing NRMT1 methylates CENP-A and it again shows that the antibody is specific to methylated CENP-A.

We depleted NRMT1 using shRNA in HeLa cells stably expressing a CENP-A-LAP (localization and purification tag containing GFP) construct. After NRMT1 shRNA expression, NRMT1 was reduced to less than 10% of control shRNA treated cells (Fig.1F). CENP-A methylation was completely lost after NRMT depletion, as shown by western blotting (Fig.1F) and immunofluorescence (Fig.1G,H), without affecting total CENP-A level. This demonstrates that NRMT1 methylates CENP-A both *in vitro* and *in vivo*.

CENP-A methylation is independent of its phosphorylation

We made different mutations at the N-terminal end of CENP-A that were designed to render CENP-A resistant to NRMT1 methyltransferase. Wild type sequence 'GPRRRRS—' was changed to AGPRRRS (MT1), GKRRRS (MT2), GPQQRS (MT3), GPQRRRS (MT4), GPQQRRRS (MT5) or GPQRRS (MT6) (Fig.2A). To verify that these mutations eliminated the ability of NRMT1 to methylate CENP-A, these mutants were

expressed as fusion protein as described in fig.1D and 2A. After purifying the proteins with Nickel beads, the 6His tag was removed by Factor X cleavage. The purified and cleaved proteins were used as substrate for our NRMT filter-binding assay. One microgram of substrate was mixed with NRMT1 and radiolabelled SAM was used as the methyl donor. The results show that,all other mutants, except mutants 4 and 5, were not methylated by NRMT1 (Fig.3B). MT4 and 5 are partially methylated (Fig.3B). Since serine 7 is close to the mutation site, we asked whether these mutations affect serine 7 phosphorylation by Aurora B. We did an *in vitro* kinase assay with recombinant Aurora B (kindly provided by Prof. Stukenberg). Aurora B phosphorylates all mutants except MT3 and MT6 (Fig.2C). However, mutants MT4 and MT5 are better substrate for Aurora B. Hence we selected MT1, 2 and 3 for our further studies. Mutant 3 is neither methylated nor phosphorylated at S7 because this mutation abrogates Aurora B consensus site.

Figure 1
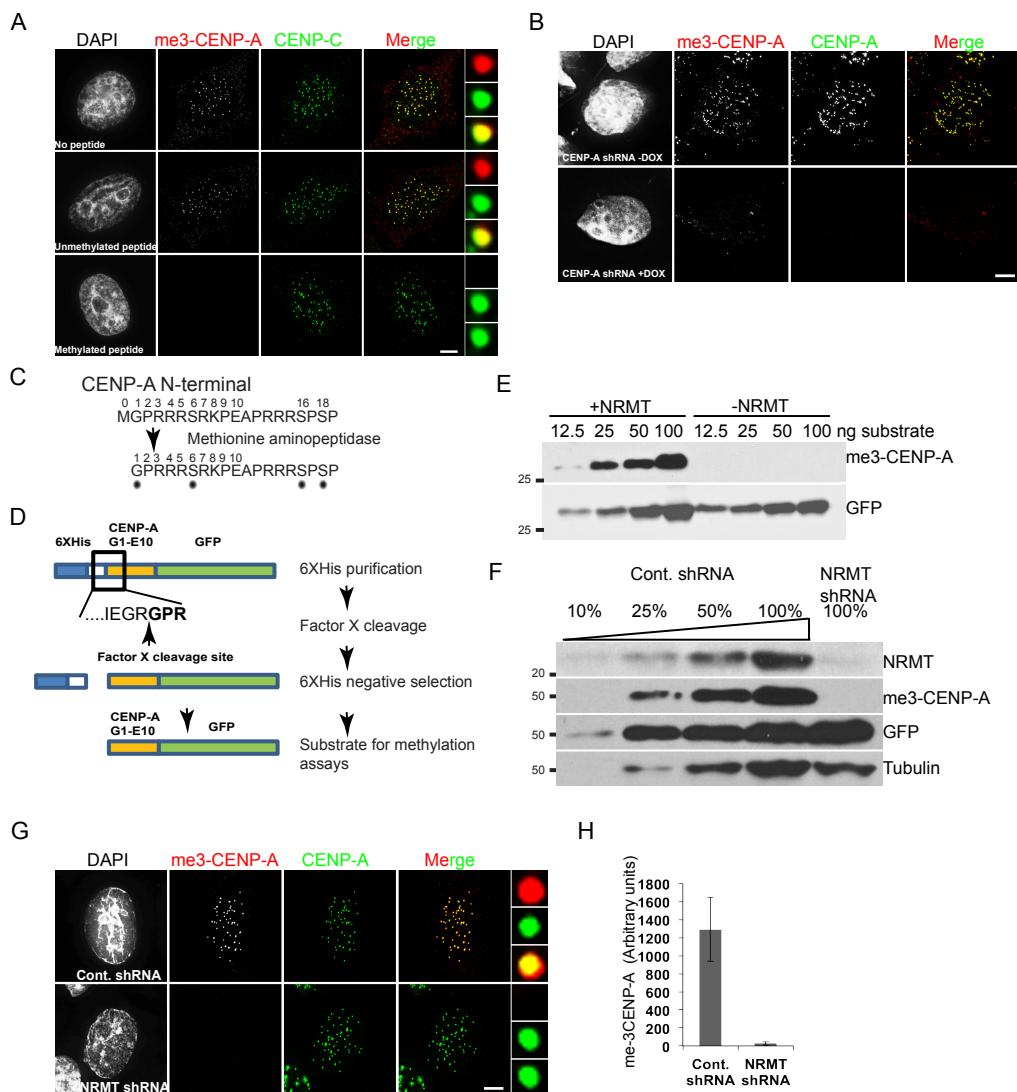
Figure 1. NRMT1 methylates CENP-A *in vitro* and *in vivo* (A). Peptide blocking experiment showing anti me3-CENP-A antibody specifically blocked by methylated peptides. (B). CENP-A knockdown followed by Immunofluorescence analysis in HEK cells using me3-CENP-A antibody, (C). Cartoon of the CENP-A N-terminal tail amino acids showing methionine aminopeptidase cleavage and removal of methionine. Dotes represent post-translationally modified amino acids, (D) Purification scheme for CENP-A N-terminal

tail 10 amino acids without beginning methionine. Proteins expressed in bacteria purified with Ni-NTA columns and then cleaved with Factor X this expose glycine for modification. 6XHis tag along with Factor X recognition site was removed by negative selection with Ni-NTA column and the eluate used as substrate for methylation reactions, (E) *In vitro* methylation of CENP-A N-terminal tail. Purified CENP-A tail treated with NRMT and western blotted for me3-CENP-A antibody showing NRMT1 methylates CENP-A, (F). Knockdown of NRMT1 followed by western blotting in HeLa cells showing loss of CENP-A α-N trimethylation, (G). Immunofluorescence analysis in NRMT1 depleted HeLa cells using me3-CENP-A antibody showing loss of CENP-A methylation, (F). Quantitation of methylated CENP-A in NRMT1 depleted cells cells.

We made CENP-A methylation mutants fused with a LAP tag in the pcDNA3-FRT-LAP mammalian expression destination vector. In addition, we also made single and combinatorial alanine substitution mutations of S7, S16, and S18. Stable cell lines were established in HeLa T-Rex Flp-IN cell lines (now on HeLa T-Rex). Lysates from these cells were used for western blotting using anti phospho-S7 antibody (Fig.2D), me3-CENP-A (Fig.2E) and anti phospho-S16;18 antibodies (Fig.2F). As expected, MT3 and S7A mutants were not phosphorylated at S7 (Fig.2D). Immunoblot of non-phosphorylated

Figure 2
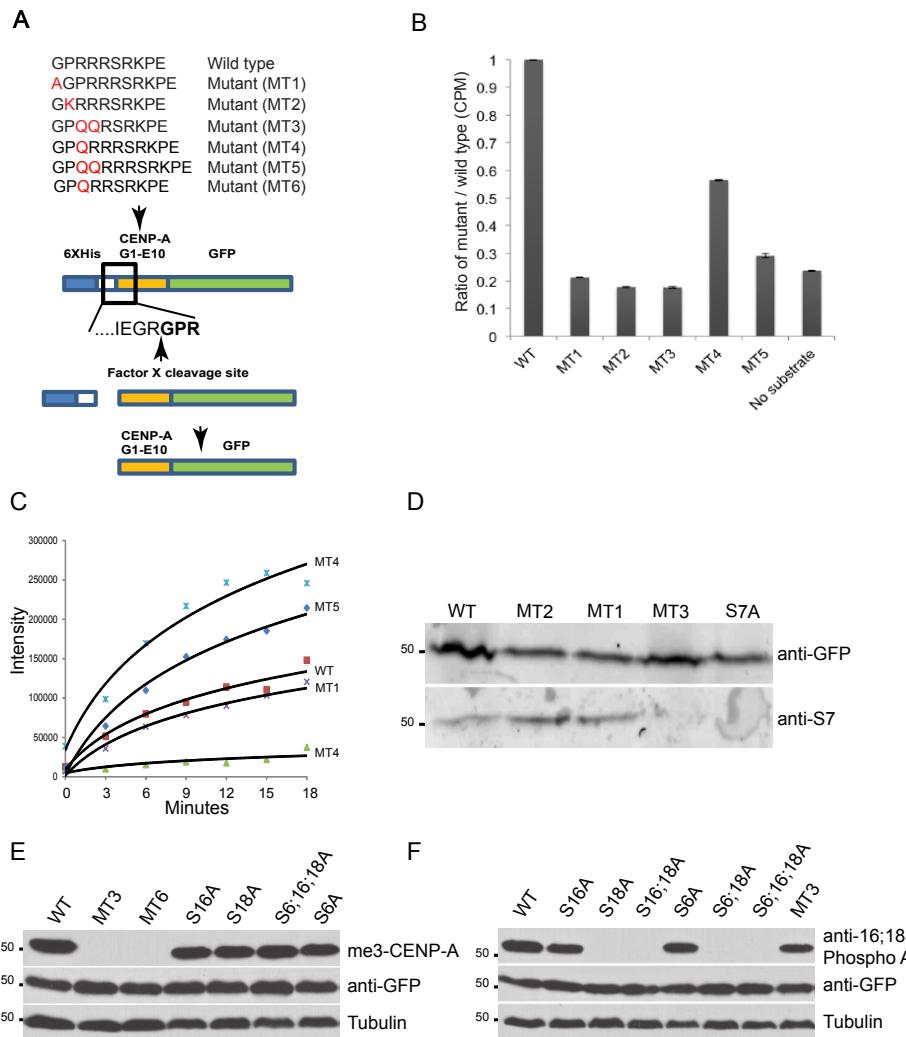
Figure 2. CENP-A methylation is independent of other known CENP-A tail post-translational modifications (A). Schematic of the 2-10 amino acids of the CENP-A tail fused with GFP on the C-terminus and 6XHis on the N-terminus. Factor X cleavage exposes the N-terminal glycine for modification by NRMT. Different mutation made at the N-terminal also shown, (B). *In vitro* NRMT methylation assay. The factor X cleaved CENP-A tail was treated with NRMT in the presence of ^{3}H-S-adenosyl-methionine. A filter-binding assay was used to determine the incorporation of radioactive methyl groups. Mutants 1 to 3 were not methylated, (C). Kinase assay using Aurora B enzyme and CENP-A wild type and mutant substrates. Graphs showing amount of phosphorylation at different time points. Mutant

1 phosphorylated similar to wild type where as mutant 3 not phosphorylated. Mutant 4 and 5 were more phosphorylated. (D). CENP-A wild type and mutants were western blotted for S7 phosphorylation. Mutant 3 and S7A were not phosphorylated (E,F). CENP-A S16 and S18 phosphorylations are independent of methylation. CENP-A methylation and phospho mutants were blotted for me3-CENP-A and phospho antibodies respectively. Me3-CENP-A antibody detected all phosphorylation mutants and anti-16;18 phospho antibody detected methylation mutant MT3, suggesting phosphorylations and methylation are independent events.

CENP-A constructs using the me3-CENP-A specific antibody showed that methylation did not require phosphorylation of the CENP-A tail. (Fig.2E). Similarly, the S16;18 phospho-antibody detected methylation mutants showing these phosphorylation did not require methylation (Fig.2F). Similarly, CENP-A's phosphorylations are not affected by loss of CENP-A methylation. The results show that CENP-A is methylated irrespective of other modifications, indicating all these events are independent (Fig.4).

Figure 3
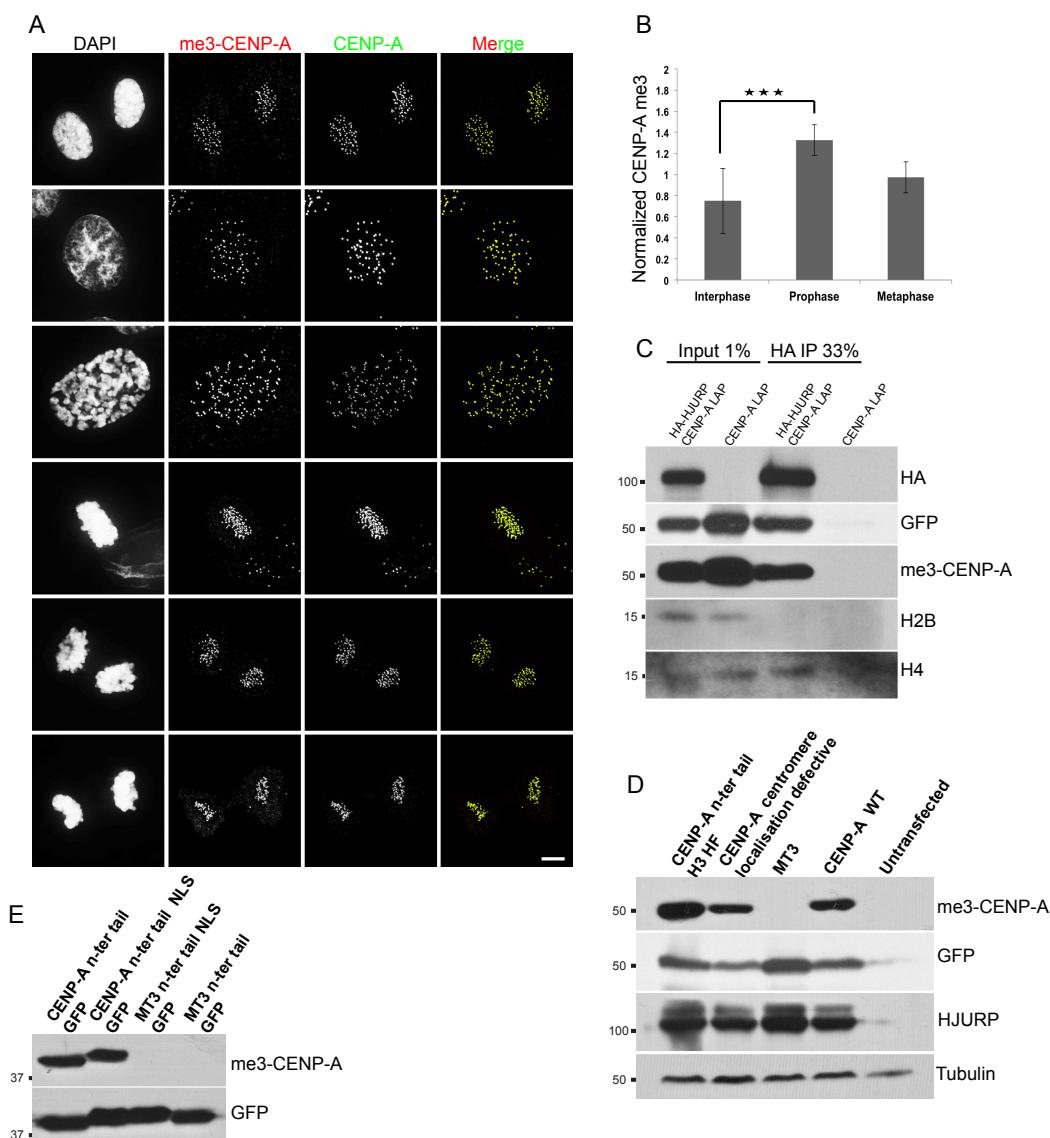
Figure 3. CENP-A methyalted throughout the cell cycle and the centromere localization not required for its methylation (A). Cell cycle distribution of methylated CENP-A at the centromeres, (B). Quantitation of the methylated CENP-A at the centromeres showing a significant increase in methylated CENP-A during prophase. (C). Pre-

nucleosomal CENP-A is methylated. CENP-A (GFP) was co-transfected with HA-HJURP and IP was done with anti-HA antibody in chromatin free extract and western blotted for me3-CENP-A, The lack of H2B in the IP fraction and presence of H4 indicating the pulled down CENP-A is pre-nucleosomal. (D). Centromere localization is not required for CENP-A methylation. CENP-A tail fused with H3.1 histone fold (H3HF) and a CENP-A centromere localization defective mutant methylated *in vivo*, (E). Methylation of CENP-A is independent of its histone fold. The cells were transfected with CENP-A tail fused with GFP and western blotted for me3-CENP-A.

CENP-A is methylated throughout the cell cycle and centromere localization is not required for its methylation

To determine the cell cycle phase at which CENP-A is methylated we stained randomly cycling U2OS cells with me3-CENP-A antibody. We found that CENP-A was methylated throughout the cell cycle. In order to identify changes in the amount of methylated CENP-A at centromeres we compared the ratio of methylated to total CENP-A, to take into account any changes in total CENP-A levels. Using this measure we observed an increase in me3-CENP-A during prophase (Fig.3A,B). We confirmed the prophase increase in CENP-A amino terminal methylation in the colorectal cancer cell line, HCT116. Given the timing of the CENP-A methylation increase, we hypothesize that increased methylation may be required for accurate segregation of chromosomes.

Figure 4
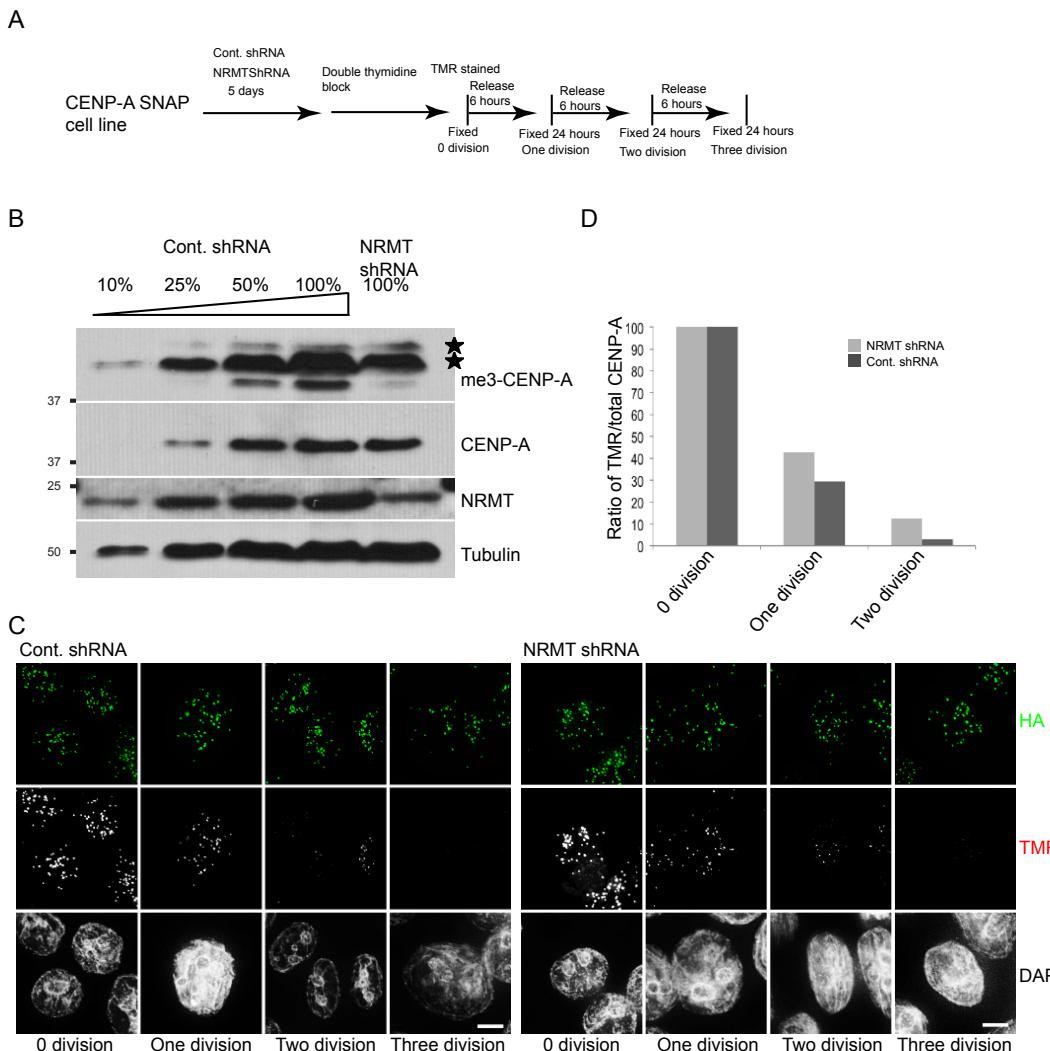
Figure 4. CENP-A methylation not required for its stability (A). Schematic diagram of the experiment. CENP-A SNAP cell lines were stably integrated with NRMT shRNA. Cells were TMR stained after double thymidine block and released to different time points (B). Western blots showing NRMT depletion and loss of CENP-A methylation. Stars indicate nonspecific bands, (C). Immunofluorescence analysis showing total CENP-A (HA) and TMR stained CENP-A after consecutive cell division, (E). Quantitation of the immunofluorescence analysis, ratio of the TMR/total CENP-A showed. CENP-A's stability was not compromised after NRMT1 knockdown.

Additional experiments will be conducted in year 2 and 3 of this proposal to deduce the relevance of this increased CENP-A methylation during the beginning of mitosis.

To determine whether CENP-A is methylated before it is deposited at the centromere, we compared pre-nucleosomal and nucleosomal fractions from cells co-transfected CENP-A-LAP and HA tagged HJURP. The chromatin free cell lysate was prepared using hypotonic lysis and then HJURP was pulled down using HA antibody. Pre-nucleosomal CENP-A forms a complex with its chaperone HJURP and histone H4. The IP shows an absence of histone H2B and presence of histone H4, clearly demonstrating we pulled down pre-nucleosomal CENP-A (Fig.3C). The western blot using me3-CENP-A antibody shows pre-nucleosomal CENP-A is methylated (Fig.3C). We transfected a mutant CENP-A that does not interact with HJURP and hence is not localized at the centromere and a construct encoding the N-terminal tail of CENP-A fused with H3 histone fold domain (H3HF). Both these mutants were methylated (Fig.3D). Moreover when a construct expressing the CENP-A N-terminal tail fused with GFP (CENP-A N-terminal GFP) was transfected into cells it was also methylated. Indicating centromere localization of CENP-A is not required for its methylation (Fig.3E).

Methylation not required for CENP-A nucleosome stability

To determine whether CENP-A methylation is required for nucleosome stability, we conducted an in vivo pulse-chase experiment using SNAP-tag as described previously[8]. The SNAP tag is a modified variant of the suicide enzyme O^6-alkylguanine-DNA alkyltransferase that irreversibly modifies (and inactivate) itself through acceptance of the cell-permeable guanine derivative O^6-benzylguanine (BG, non-fluorescent; or TMR-star, fluorescent). This system allows labeling of SNAP fusion proteins *in vivo*. We used pulse labeling with this methodology to determining CENP-A turnover specifically at centromeres with or without NRMT1 knockdown. We established cell lines stably expressing both centromere-localized CENP-A–SNAP and either control shRNA or NRMT1 shRNA plasmids. The cells were synchronized at the G1–S boundary by double thymidine block. After second thymidine block CENP-A was pulse-labeled with TMR-*Star* and were chased for up to two cell cycles (Fig.4A). The western blot shows approximately 90% of NRMT1 was depleted and CENP-A methylation was reduced significantly (Fig.4B). After first cell division, the centromere TMR-star labeled CENP-A-SNAP was reduced to 40% in NRMT1 knockdown samples and 35% in control shRNA transduced cells. After a second cycle of DNA replication, the previously labeled centromere-bound CENP-A–SNAP was reduced to less than 10% and 5% of its initial level in knockdown and control samples respectively. These reductions are consistent with dilution of CENP-A nucleosomes during DNA replication. Since we observed no difference in the

reduction of CENP-A between control and NRMT shRNA treated cells, these preliminary results suggest CENP-A's stability is not compromised the lack of CENP-A methylation.

CENP-A depletion causes multipolar spindle formation in a p53 dependent manner

CENP-A histones epigenetically determine the centromere, which is where kinetochore forms during mitosis and accurately segregate chromosomes. Overexpression of CENP-A leads to its mislocalisation and missegregation of chromosomes[9]. Similarly loss of CENP-A also causes missegregation of chromosomes by improper kinetochore

Figure 5
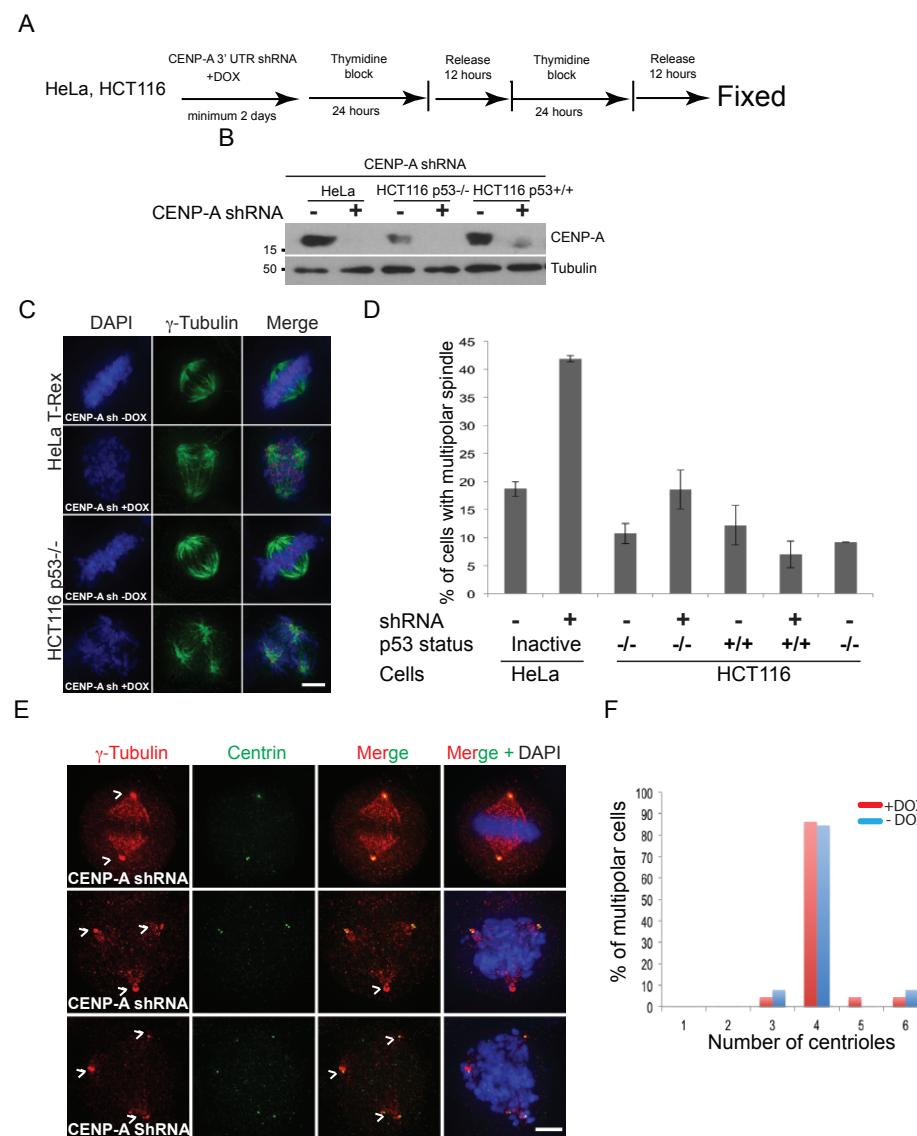
Figure 5. CENP-A knockdown leads to multipolar spindle in p53 inactive cells (A). Schematic diagram of the experiment. CENP-A shRNA was induced for two days before starting the double thymidine block and release. Cells

were fixed 12 hours after second release, (B). Western blots show depletion of of CENP-A after induction of shRNA using doxycycline (DOX), (C) CENP-A knockdown causes multipolar spindle. Cells were stained for α-tubulin after CENP-A knockdown, (D). Percentages of the multipolar cells. HeLa and HCT116 p53-/- cells show higher percentages of multipolar cells, (E). Examples of bipolar and multipolar cells stained for γ-tubulin and centriole marker centrin showing centrosomes without centriole. The arrows in the first column indicate centrosomes and in the third column the centrosomes with no centriole or one centriole (F). Number of centrioles in multipolar cells. The majority of cells have only four centrioles both in control and CENP-A knockdown samples.

Figure 6
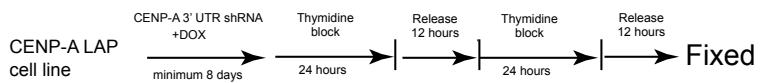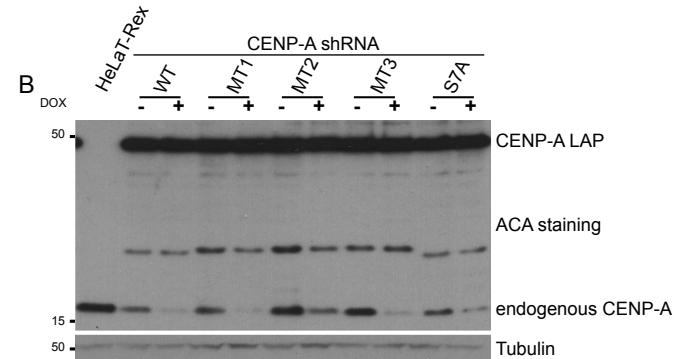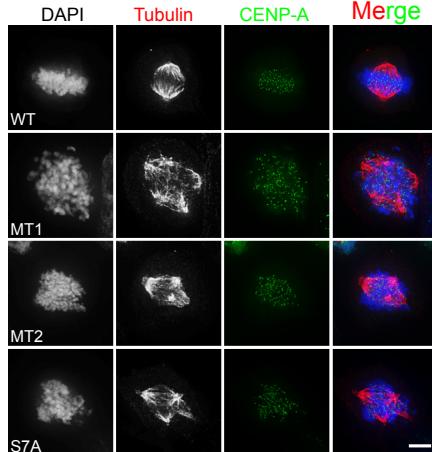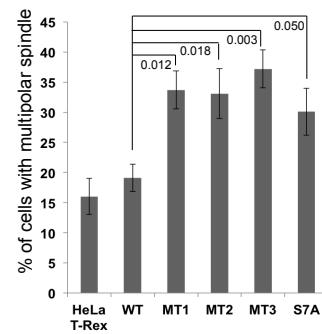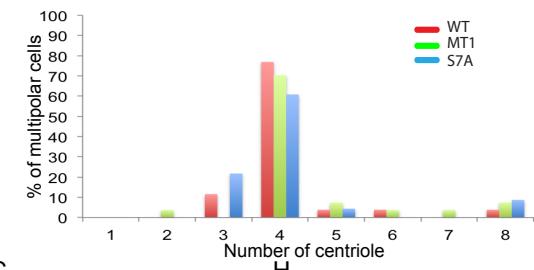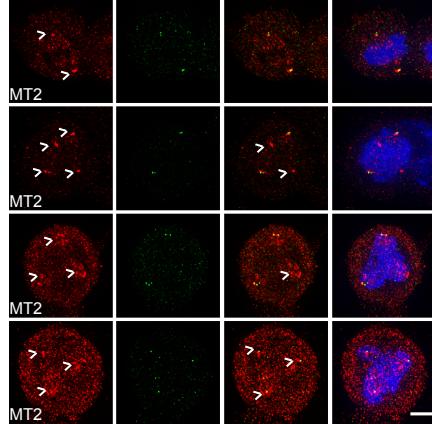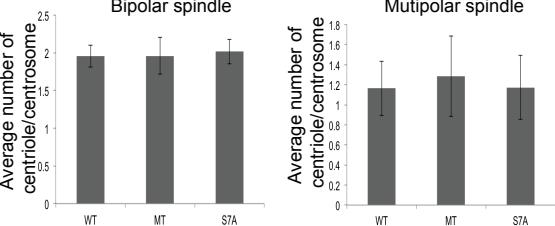
Figure 6. Loss of CENP-A methylation causes multipolar spindle in HeLa cells (A). Schematic diagram of the experiment. CENP-A shRNA was induced for 8 days before starting the double thymidine block and release. Cells were fixed 12 hours after second release, (B). Western blots of the CENP-A-LAP wild type and methylation mutant stable cell lines stably integrated DOX inducible CENP-A 3'UTR shRNA before and after DOX induction. The membrane was probed with ACA showing endogenous CENP-A reduced after knockdown, (C). Methylation deficient mutants (MT 1,2 and 3) of CENP-A causes multipolar spindle (D). Quantitation of the multipolar cells. Multipolar cells were significantly different from both parental cells and wild type replaced cells. S7A mutant also causing multipolar spindle, (E). Examples of γ-tubulin stained multipolar cells. Majority of cells has one or more centrosome without centrioles. The arrows in the first column indicate centrosomes and in the third column depicting centrosome with one or no centriole, (F). Number of centrioles per multipolar cells are shown. Majority of cells have only four centrioles. Indicating lack of centriole duplication as a mechanism to form multipolar cells, (F,G). Average number of centrioles per centrosome in multipolar and bipolar cells. Multipolar cells have less centriole per centrosome.

formation[10]. In the presence of p53, loss of CENP-A induces senescence in immortalized cells[10]. To determine the phenotype of CENP-A loss in tumor cells, we depleted CENP-A in p53 wild-type and null isogenic HCT 116 colorectal cancer cells[11] as well as in a HeLa cell line using shRNA against the CENP-A 3' UTR. P53 is inactivated in HeLa cells due to expression of the HPV E6 protein. We found CENP-A levels were significantly reduced after shRNA induction by addition of doxycycline (Fig.5A,B). Phenotypically, we found that CENP-A depletion caused a significant increase in the number of cells with multipolar spindles in the HeLa T-Rex line (Fig.5C,D). In HCT116 cells, depletion of CENP-A caused more cells to posses multipolar spindles in p53 null cells but not in p53 wild-type cells (Fig.5D). Both these results show that depletion of CENP-A causes multipolar spindle formation in p53 inactive or null cancer cells. We also checked whether the multipolar spindles were formed because of centriole duplication or centrosome splitting (Fig.5E,F). Usually failure in cytokinesis

causes centriole duplication. We found that the majority of multipolar cells have only four centrioles (Fig.5F). And also majority of multipolar cells showed at least one γ-tubulin stained centrosome like structures without centriole in it. In some cells centrioles split between centrosome having one centriole per centrosome instead of two. All these results indicated that multipolar spindles induced by CENP-A depletion or expression of methylation constrcuts were formed by centrosome splitting, and not by a failure in cytokinesis or centrosome reduplication.

We determined whether CENP-A depletion causes any increase in lagging chromosome during telophase. As we expected, there was increased number of lagging chromosomes in both p53-/- and p53+/+ isogenic HCT116 cells when CENP-A was depleted. p53+/+ cells showed dramatic increase in lagging chromosomes upon CENP-A depletion. In p53-/- cells, the overall percentage of cells showing lagging chromosomes was initially high and it was shifted upward after CENP-A knockdown (Fig.7E,F). In p53-/- cells showed a significant increase in multipolar spindle formation; however, p53+/+ cells did not, perhaps dues to p53 dependent mechanism known to suppress multipolar spindle formation[12] (Fig.7C-H). All these show that CENP-A is essential for maintaining proper chromosome segregation and bipolar spindle.

CENP-A methylation is required for bipolar spindle formation and error free chromosome segregation

To dissect the function of CENP-A methylation we made CENP-A methylation mutant stable cell lines in HeLa T-Rex (Fig.6) and HCT116 p53-/- and p53+/+ cells (Fig.7). We have made stable cell lines in HCT116 cells by viral integration and the cells expressing

the construct were selected. For HeLa T-Rex, we have used the Flp-In method to generate stable cell lines. We then stably integrated doxycycline inducible 3'UTR targeting CENP-A shRNA using lentivirus. Because the shRNA targets the 3'UTR, it selectively depletes endogenous CENP-A, but not our exogenous mutants. Eight days after starting doxycycline-mediated induction of shRNA, the cells were plated on coverslips and we did a double thymidine block and release experiment. 12 hours after

Figure 7
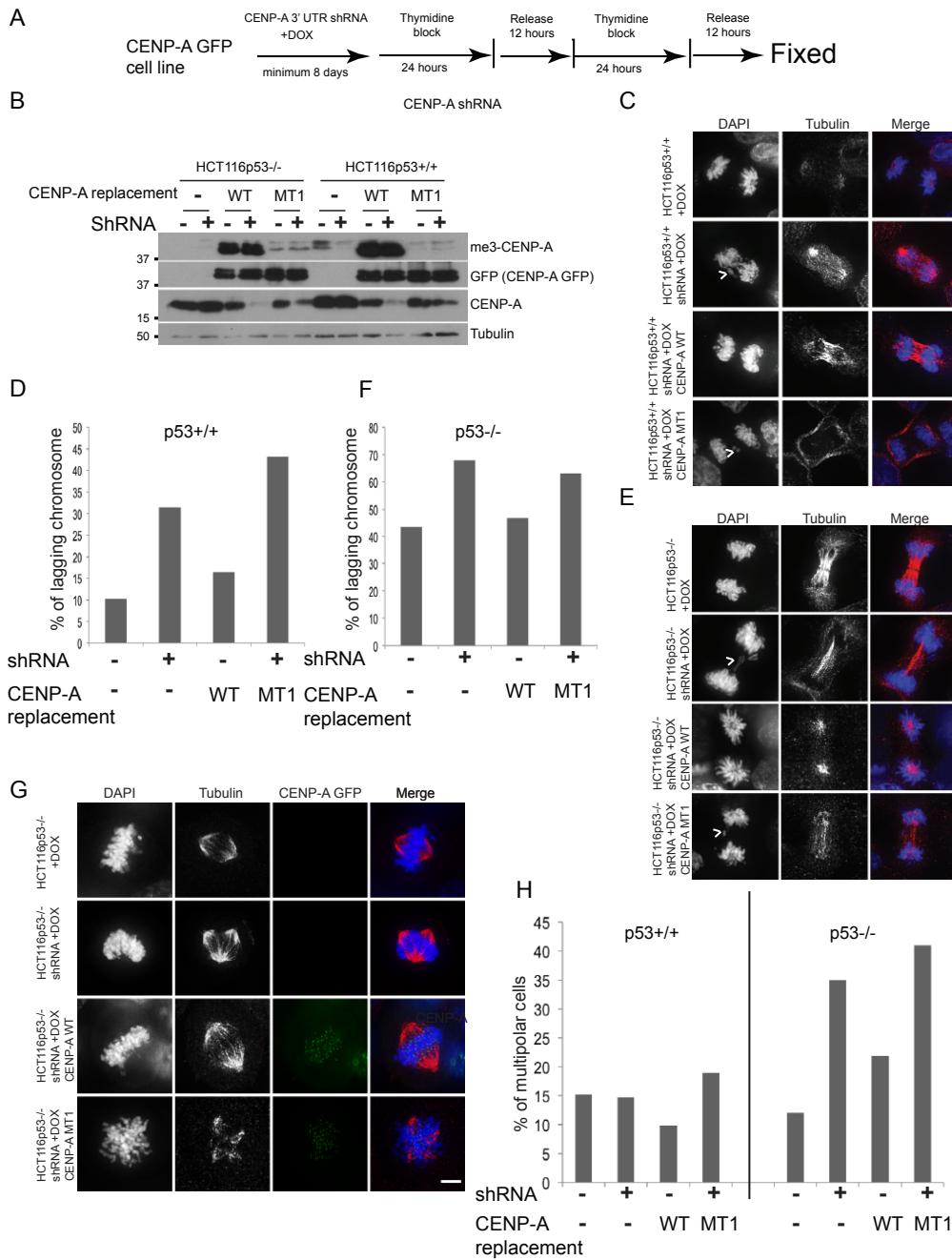
**Figure 7. Loss of CENP-A methylation causes lagging chromosomes in p53 +/+ colorectal cancer cells and multipolar cells in p53-/- colorectal cells** (A). A schematic diagram of the experiment, (B). Western blots of the knockdown and replacement

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experiments. Endogenous CENP-A level reduced in the replaced cells and was further reduced in knockdown cells, (C) Knockdown and replacement experiment of CENP-A methylation mutant. Figure showing lagging chromosomes (arrows) in CENP-A knockdown HCT116 p53+/+ cells and was rescued by wild type CENP-A but not mutant (MT1), (D). Graph showing the percentage of cells having lagging chromosomes (arrows) in HCT116 p53+/+ cells in knockdown and replacement experiment of CENP-A methylation mutant, (E). Cells showing lagging chromosomes in CENP-A knockdown HCT116 p53-/- cells and was rescued by wild type CENP-A but not mutant (MT1), (F). Graph showing the percentage of cells having lagging chromosomes, (G). Cells showing multipolar spindle after CENP-A knockdown replacent with methylation mutant (MT1) in HCT116 p53-/-, (H). Percentage of multipolar cells in p53-/- and p53+/+ HCT116 colorectal cancer cells. In p53-/- cells the CENP-A knockdown leads to increased multipolar cells and was partially rescued by wild type CENP-A but not methylation mutant (MT1).

the second release cells were fixed and stained for tubulin and DAPI (Fig.6A,7A). At the same time we also harvested cells for western blotting and the western blot showed a significant depletion of endogenous CENP-A (Fig.6B,7B). We found that the multipolar spindle formation induced by CENP-A depletion (Fig.5C,D) was rescued by CENP-A wild type replacement, but not by methylation mutants and S7A mutant (Fig.6C,D). Interestingly, mutant 3, which is neither alpha-N-methylated nor phosphorylated at S7, showed the highest percentage of multipolar cells. Showing a slight additive effect (Fig.6C,D). Similar to CENP-A depleted cells, the mutant replaced cells also showed centrosome splitting, where two daughter centrioles are found in different poles (Fig.6E,F,G). The majority of multipolar cells have the expected four centrioles, with some centrosomes containing only one centriole or none (Fig.6E).

Similar to the HeLa cells HCT116 p53-/- cells, methylation mutant cells showed increased number of multipolar cells compared to the CENP-A wild-type rescue (Fig.7G,H). However, the percentage of p53+/+ HCT116 cells with multipolar spindles was similar in both CENP-A wild type and mutant cells (Fig.7G,H). Compared to our previous experiment, where we depleted CENP-A only for four days, here we found an increase in multipolar spindle (Fig.5D,6H). Indicating a longer knockdown cause more spindle defects in HCT116p53-/- cells.

We also analyzed chromosome segregation defects in these cells. Any defect in chromosome segregation during telophase was counted. We found a significantly higher percentage of cells showing lagging chromosomes when we depleted CENP-A and replaced with methylation mutant CENP-A in p53+/+ HCT116 relative to rescued with wild type CENP-A (Fig. 7C,D). In p53-/- HCT116 cells, though the overall percentage of lagging chromosomes was initially higher than p53+/+ cells, the depletion and replacement with the methylation resistant CENP-A mutant showed an increase in lagging chromosomes compared to the wild type CENP-A rescue (Fig.7 E,F). Therefore we conclude that CENP-A amino-terminal methylation is required for proper chromosome segregation and its loss leads to aneuploidy in colorectal cancer cells.

Loss CENP-A methylation causes uncontrolled proliferation
To determine the role of CENP-A in cell viability and proliferation, we performed a colony formation assay using the HCT116 wild type and p53 null cells where we depleted CENP-A. We have used the double stable cell line of CENP-A 3'UTR shRNA and either CENP-A wild type or CENP-A methylation mutant 1 for our knockdown replacement experiment. The CENP-A methylation mutant cell line formed bigger colonies in p53-/- cells when compared to wild type cells after 8 days of CENP-A depletion. We also found significantly bigger colonies in p53+/+ cells. These data suggest that, in the absence of p53, the loss/reduction of CENP-A methylation provides proliferative advantage and thus promote tumorigenesis. Whereas, in the presence of

p53, the chromosome segregation defects suppressed by p53 but eventually leads to bigger tumors.

Discussion:

The post-translational methylation of alpha-amino groups was first discovered over 30 years ago[3]. However, its biological function remains obscure except in the case RCC1 (Ran guanine nucleotide-exchange factor) where it is required for its localization into chromosomes[13]. Here we identified a novel function of alpha-N-methylation. We found that alpha-N-methylation of CENP-A is required for maintaining genomic stability and its loss leads to aneuploidy.

We have demonstrated CENP-A is methylated throughout the cell cycle and methylated CENP-A increases during prophase. CENP-A is methylated prior to its deposition. However, we cannot rule out the possibility that a fraction of CENP-A is methylated after its deposition at the centromere. The increase in methylated CENP-A during prophase may suggest such a possibility. The increased methylation prior to metaphase is consistent with a role of methylation in chromosome segregation. Although we do not know the direct consequences of CENP-A methylation as of yet, the next two years of support form the DOD will allow us to determine the molecular interaction that underlie this effect.

Figure 8
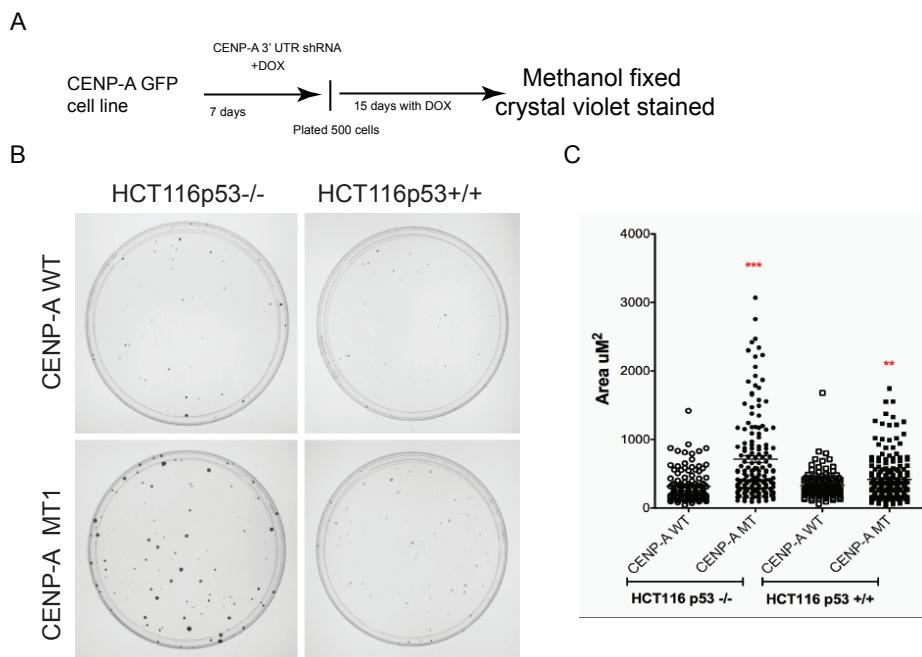
Figure 8. Loss of CENP-A methylation causes increased cell proliferation and survival in a p53 dependent manner (A). A schematic diagram of the experiment. CENP-A-GFP wild type or mutant constructs were stably integrated along with DOX inducible CENP-A 3'UTR shRNA in p53+/+ and p53-/- HCT116 cells lines. Dox was added to the double stable cells for seven days and 500 cells from each experiment was plated for the colony formation assay. The cells were grown under continued presence of DOX. After 15 days cells were methanol fixed and stained with crystal violet, (B). Representative pictures of the colony formation assay. (C). Quantitation of the area of the colonies. Area of the colonies significantly different between CENP-A wild type and mutant both in p53-/- (<0.0001) and p53+/+ (0.0091) HCT116 cells.

Reduction in CENP-A level increases the propensity of cells to form multipolar spindle pole and lagging chromosome in p53 null colorectal cancer cell HCT116. Whereas, in isogenic p53+/+ cells there is no significant increase in multipolar spindle formation after CENP-A knockdown; however there is a dramatic increase in lagging chromosome. Moreover, we also found increased multipolar cells in p53 inactive HeLa cells, suggesting that reduction of CENP-A causes aneuploidy either by multipolar spindle formation or by forming lagging chromosomes or both. It has been previously shown that NRMT1 depletion cause multipolar spindle in HeLa cells[6]. We wondered whether this effect is through a hypomorphic CENP-A that is methylated by NRMT1[3]. In our shRNA depletion replacement experiment, cells rescued with wild type CENP-A had similar percentage of multipolar cells as that of parental cell lines both in colorectal cancer cell HCT116 p53-/- and HeLa. But rescue with the methylation mutant CENP-A showed significantly increased multipolar spindle in p53-/- HCT116 and p53 inactive HeLa cells, suggesting CENP-A methylation mutants are hypomorphic.

We also analyzed chromosome missegregation defects and found that there is an increase in lagging chromosomes compared to wild type irrespective of p53 status. The lagging chromosomes occur when kinetochores form improper microtubule attachments. We are currently looking for changes in centromere and kinetochore associated proteins in these cells to determine any defects in their localization. In normal cells it may be possible that loss of CENP-A methylation drive chromosome missegregation and when p53 is mutated it induce tumorigenesis or vice versa. It is important to look the methylation status of CENP-A in colorectal cancer tissues to see whether methylated CENP-A is reduced during tumorigenesis and tumor progression.

We also found further evidence that CENP-A methylation has a protective effect in tumorigenesis. In the colony formation assay when endogenous CENP-A is replaced with wild type or methylation mutant by depletion and replacement, the mutants formed larger colonies. This effect was more pronounced in p53-/- HCT116 cells. All these results suggest a role of CENP-A methylation in normal cell division and proliferation. Our model suggest that overexpressed CENP-A, which is very common in colorectal cancer may not be fully methylated and that may cause chromosome instability and multipolar spindles. This may lead to more aggressive tumors. We have made exciting progress in the proposed study and continuation of this study is necessary to fully understand the function of alpha-amino group methylation of CENP-A. In the next year we will focus on to elucidate the mechanism of the chromosome segregation defects in the methylation mutants. We will look alteration in the localization of the centromere and kinetochore associated complexes in methylation mutants. We will also look at the status of CENP-A methylation in colorectal tumor samples to see its direct association with cancer progression and survival.

Materials and methods:
In vitro **methylation assays:** 6XHis tagged Human NRMT1 (Gift from Ian Maccara) was purified from E- *coli* and used for the methylation assays. CENP-A initial 2-10 amino acids were purified as a fusion protein. 6XHis followed by Factor X cleavage site and then CENP-A ten N-terminal amino acids starting from second amino acid Glycine fused with GFP's N-terminal. The fusion proteins were expressed from a modified pET15b (Novagen) expression vector in BL21 *E. coli* and purified on Ni-NTA beads and

then cleaved using Factor X (Sigma-Aldrich). The cleaved proteins were then negatively selected with Ni-NTA column and the eluate used as substrate for methylation assays. Reactions (50 µl) were performed in MTase buffer (50 mM Tris, 50 mM potassium acetate, pH 8.0), using 3.0 pmol of recombinant NRMT plus 1µg of purified substrates. The reaction was incubated for 2 h at 30 °C after addition of 1 µl of ^{3}H-SAM (0.55 µCi µl^{-1}). Reactions were filtered through nitrocellulose, washed with 50 mM sodium bicarbonate and subjected to scintillation counting. For western blotting analysis, cold SAM was used instead of ^{3}H-SAM.

shRNA mediated knockdown of NRMT1 and CENP-A: The human and mouse NRMT lentiviral shRNAmir pGIPZ constructs were obtained from Ian Maccara's lab. The targeting sequence of the shRNA against NRMT is AGAGAAGCAATTCTATTCCAAG; and the control sequence is CCCTGCCAGACAGTACCAATTA. To make virus, 2.5 × 10^6 293LT cells were calcium-phosphate-transfected with 20 µg of the NRMT pGIPZ plasmid, 6 µg of the vesicular stomatitis virus coat protein plasmid (pMD2G), and 15 µg of packaging plasmid (psPAX2)[6]. Viral supernatants were collected after 48 h and infected HeLa T-Rex or HCT116 cells. After 3 days, 2 µg ml^{-1} puromycin was added to select transduced cells. For CENP-A knockdown, doxycycline inducible shRNA targeting 3'UTR on pTRIPZ constructs was used. The viruses were made as described for NRMT1 and infected HeLa T-Rex and HCT116 cells. CENP-A equally distributes between daughter chromosomes during DNA replication. It takes at least four cell divisions to reduce CENP-A below 10% level. For CENP-A knockdown, shRNA was induced two days before starting double thymidine block and release so that the cells undergo approximately four cell divisions to get effective knockdown.

For knockdown and replacement experiments in HCT116 cells, CENP-A wild type and mutants were cloned into pBABE retrovirus vector by cold fusion. CENP-A fused with GFP on the C-terminus. The viruses were packaged by transfecting into 293 GP cells along with VSVG plasmid. The viruses were collected after 3 days. For making double stable cell lines, the cells were co-infected with CENP-A retrovirus and CENP-A 3'UTR shRNA lentivirus. The double stable cells were selected using 6 µg ml^{-1} Blasticidin and 2 µg ml^{-1} puromycin. CENP-A was also cloned into modified pcDNA3/FRT-LAP destination vector kindly provided by Stukenberg lab. HeLa T-Rex cells were transfected with CENP-A wild type or mutants along with FLP-recombinase. The stable cells were selected with hygromycin. The stables cells were then infected with doxycycline inducible CENP-A shRNA virus to establish double stable cell lines. For knockdown replacement experiments shRNA was induced 8 days before starting double thymidine block and release to get effective endogenous CENP-A depletion.

Peptide blocking: Cells were pre-extracted for 3 minutes using 0.1% triton followed by fixation in 4% formaldehyde for 10 minutes. The cells were then blocked in 1XPBS containing 2% BSA, 2% fetal calf serum and 0.1% triton for one hour. Meanwhile me3-CENP-A antibody was pre-incubated with methylated or unmethylated CENP-A N-terminal peptide at a concentration of 10X peptide to 1X antibody for 30 minutes at room temperature. The antibody then centrifuged and the supernatant used for staining as described in immunofluorescence section.

Immunofluorescence: Cells were either pre-extracted with 0.1% triton in 1X phembuffer (60mM PIPES, 25mM HEPES, 10mM MgCl2) for 3 minutes and then fixed in 2% formaldehyde for 10 minutes or fixed in 2% formaldehyde for 10 minutes and then permeabilized in 0.1% triton for 5 minutes. The cells were then blocked in 2% BSA and 2% fetal calf serum with 0.1% triton in 1XPBS. It was incubated with primary antibodies one hour and then secondary antibody was added for one hour. All washes between each step were done in 1XPBS + 0.1% triton. DNA was stained with DAPI and were mounted in prolong. Cells were examined and images were acquired using a Delta Vision optical sectioning deconvolution instrument (Applied Precision) using a X100 oil-immersion Olympus objective lens connected with Photometrics CoolSNAP HQ^2 camera and Softwrox acquisition software. Images were deconvolved and presented as maximum stacked images. For staining γ-tubulin and centrin, cells were fixed in ice-cold methanol on ice for 3 minutes and then proceeded with antigen blocking as described above. The antibodies used for immunofluorescence were: mouse anti-CENP-A (1:1000), Rabbit anti-me3CENP-A (1:200), mouse anti-CENP-A (1:1000), Rabbit anti-CENP-T (1:1000), Rabbit anti- g-tubulin (1:1000; Sigma), Mouse anti-centrin (1:1000; Millipore), Mouse anti-α-tubulin (1:1000).

Following antibodies were used for IP and western blotting. Rabbit anti-GFP (1:3000), Rabbit anti-H2B (1:1000), Rabbit anti-H4 (1:5000), Mouse anti-HA (1:1000), Mouse anti-tubulin (1:1000).

SNAP tagging and pulse chase experiment: CENP-A–SNAP–3XHA cell lines previously described used in this study[5]. NRMT1 or control shRNA was stably integrated into this cell lines. Cells were then double thymine blocked using 2mM thymidine. During the end of second thymidine block, the cells were labeled with 2 µM TMR-*Star* (Covalys) in complete growth medium for 60 min at 37°C. Labeling was followed by one wash each with PBS, and DMEM and incubated for 30 min, and washed with PBS prior to fixation (0 division). The cells were then released 6 hours before adding 2mM thymidine and cells were fixed after 12 hours after release (1 division). Similarly cells were released and fixed for second and third divisions. Total CENP-A was then stained with anti-HA antibody and counter stained with DAPI.

Key accomplishments:

Following are the key accomplishments of the proposed work

1. Identified NRMT1 methylates CENP-A at its α-amino terminal.
2. CENP-A is methylated throughout the cell cycle.
3. Pre-nucleaosomal CENP-A is methylated.
4. We made several methylation resistant CENP-A mutants.
5. Loss of CENP-A cause aneuploidy by forming multipolar spindle and missegregation of chromosomes.
6. α-amino tri-methylation of CENP-A ensures high fidelity of chromosome

segregation. Its loss cause multipolar spindle and lagging chromosomes in p53 null background and lagging chromosomes in the presence of p53. Both these alterations fundamentally cause aneuploidy, a form of genetic alterations prevalent (85%) in colorectal cancer.

7. We also found that methylation mutant CENP-A forms bigger colonies in colony formation assay suggesting role of methylation in controlling proliferation.

We have presented a poster regarding the role of CENP-A α-amino tri-methylation in accurate chromosome segregation and cancer during the international Keystone Cancer Epigenetics conference held in February 2014 (poster attached).

Reportable outcome:

All the eight figures in the results section will form the basis of a manuscript we are planning to write. We have found that CENP-A α-amino tri-methylation is a crucial post-translational modification in maintaining high fidelity of chromosome segregation and any defect in this modification may results in aneuploidy and cancer.

Conclusion:

We have made significant progress in elucidating the function of α-amino tri-methylation of CENP-A. We achieved several of the proposed aims. We found that CENP-A is methylated by NRMT1 and this modification persists throughout the cell cycle. We also found that α-amino tri-methylation of CENP-A is critical in orchestrating chromosome segregation and its abrogation may leads to aneuploidy and cancer. Currently, we are analyzing how CENP-A α-amino tri-methylation orchestrating chromosome segregation. We are looking at alterations in proteins associated with the centromere and kinetochore in the CENP-A mutant stable cell lines. We also want to look at the status of CENP-A tri-methylation in colorectal cancer in year 2 and 3 of this proposal. This will tell us the role of α-amino tri-methylation of CENP-A in cancer progression and survival. Further support is necessary to fully understand the function of α-amino tri-methylation of CENP-A and its role in colorectal cancer.

Meeting attended/presented

Cancer Epigenetics, Keystone symposia, February 4-9, 2014.

N-terminal α-amino trimethylation of centromere histone CENP-A required for maintaining bipolar spindle and regulated cell proliferation. Kizhakke M. Sathyan, Aaron O. Bailey, Tanya Panchenko, Donald F. Hunt, Ben E Black, Daniel R. Foltz. Cancer Epigenetics, Keystone symposia, February 4-9, 2014.

Abstract of the poster:

The deposition of histone H3 variant CENP-A marks centromere in higher eukaryotes, chromosomal loci required for accurate segregation of chromosome. It has been shown a majority of cancers exhibits chromosomal instability, however, the mechanism is unclear. Overexpression of CENP-A and its chaperone HJURP occurs in many cancers and is sufficient to cause chromosome missegregation in cell culture. The posttranslational modification (PTM) of histones resulting in the recruitment of activator and repressive complexes to chromatin is a well established and broadly used method to regulate chromatin activity by the cell. We propose that the PTMs of CENP-A have a major role in preventing chromosomal instability. Despite the identification of nearly 14 PTMs on the histone H3 amino terminus, many of which have significant functional consequences, prior to our work only a single phosphorylation on serine 7 (pSer7) of CENP-A has been described in higher eukaryotes. Recently, we have reported three new PTMs on human CENP-A N-termini using high-resolution MS: trimethylation of Gly1 and phosphorylation of Ser16 and Ser18. The nascent N-terminal residue Gly1 becomes trimethylated on the α-amino group following initial methionine removal. Here we demonstrate that the N-terminal RCC1 methyltransferase (NRMT1) methylates CENP-A both *in vitro* and *in vivo*. Even though it was identified 30 years ago, the function of N-terminal α-amino group methylation is poorly understood. Methylation occurs in the prenucleosomal as well as nucleosomal forms of CENP-A, although nucleosomal CENP-A showed higher methylation. *In vivo* as well as *in vitro* data suggest that the amino terminal tail of CENP-A is sufficient for trimethylation. We also found significant differences in methylation of centromeric CENP-A during prophase of the cell cycle. The CENP-A methylation resistant mutants show multipolar spindles, multiple centrioles and multinucleated cells indicating a failure of cytokinesis in p53 inactivated HeLa cells. We also found similar results in p53-/- HCT116 cells. The methylation mutant cells formed larger and higher number of colonies indicating uncontrolled growth in p53-/- cells, where as flatter cells in p53+/+ cells may be due to induction of senescence. We are currently analyzing how the loss of CENP-A α-amino trimethylation leads to multipolar spindles and uncontrolled cell growth. Supernumerary centrosomes are a common characteristic of cancer cells and a source of chromosome instability. Our work provides a novel link between CENP-A posttranslational modification and the generation of chromosome instability through changes in centrosome number and cell proliferation

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Appendices:

Abbreviations used

PTM   Post-translational modifications

CIN   Chromosomal instability

MIN   microsatellite instability

NRMT1   N-Terminal RCC1 Methyltransferase 1

NRMT2   N-Terminal RCC1 Methyltransferase 2

shRNA   short hairpin RNA

LAP tag   Localization and Affinity Purification tag

SNAP tag   Modified human O^6-alkylguanine-DNA alkyltransferase

SAM   S-adenosyl methionine

UTR   untranslated region

CENP-A   Centromere protein-A

ACA   Anti-centromere antibodies

α-N-me3   alpha N-terminal amino acid trimethylation